UNITED STATES PATENT AND TRADEMARK OFFICE

CERTIFICATE OF CORRECTION

PATENT NO. : 9,562,263 B2
APPLICATION NO. : 14/067620
DATED : February 7, 2017
INVENTOR(S) : Brian K. Maples et al.

It is certified that error appears in the above-identified patent and that said Letters Patent is hereby corrected as shown below:

In the Claims

Column 34, Line 63, Claim 23, after "sequence" insert -- , --

Signed and Sealed this
Twenty-sixth Day of September, 2017

Joseph Matal
Performing the Functions and Duties of the
Under Secretary of Commerce for Intellectual Property and
Director of the United States Patent and Trademark Office

Attorney's Docket No.: 30171-0025002 / ITI-001

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

First Named Inventor	:	Brian K. Maples	Art Unit	:	1637
Patent No.	:	9,562,263	Examiner	:	Angela Marie Bertagna
Issue Date	:	February 7, 2017	Conf. No.	:	4288
Serial No.	:	14/067,620			
Filed	:	October 30, 2013			

Title : NICKING AND EXTENSION AMPLIFICATION REACTION FOR THE EXPONENTIAL AMPLIFICATION OF NUCLEIC ACIDS

Attention Certificate of Corrections Branch
Commissioner for Patents
P.O. Box 1450
Alexandria, VA 22313-1450

REQUEST FOR CERTIFICATE OF CORRECTION

Please issue a certificate of correction in accordance with the attached form.

All errors to be corrected were made in printing by the Patent and Trademark Office, and no fee is believed to be due.

Apply any other necessary charges or credits to Deposit Account 06- 1050, referencing the above attorney docket number.

Respectfully submitted,

Date: March 9, 2017_____________

/Ian J.S. Lodovice, Reg. No. 59,749/
Ian J. Lodovice
Reg. No. 59,749

Customer Number 26161
Fish & Richardson P.C.
Telephone: (617) 956-5972
Facsimile: (877) 769-7945

23623696.doc

PTO/SB/44 (09-07)
Approved for use through 08/31/2013. OMB 0651-0033
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(Also Form PTO-1050)

UNITED STATES PATENT AND TRADEMARK OFFICE
CERTIFICATE OF CORRECTION

PATENT NO. : 9,562,263

APPLICATION NO.: 14/067,620

ISSUE DATE : February 7, 2017

INVENTOR(S) : Brian K. Maples, Rebecca C. Holmberg, Andrew P. Miller, Ph.D., Jarrod Provins, Richard Roth, Jeffrey Mandell and

It is certified that an error appears or errors appear in the above-identified patent and that said Letters Patent is hereby corrected as shown below:

Col. 34, line 63, claim 23, after “sequence” insert - - , - -

MAILING ADDRESS OF SENDER (Please do not use customer number below):
Fish & Richardson P.C.
P.O. Box 1022
Minneapolis, MN 55440-1022

This collection of information is required by 37 CFR 1.322, 1.323, and 1.324. The information is required to obtain or retain a benefit by the public which is to file (and by the USPTO to process) an application. Confidentiality is governed by 35 U.S.C. 122 and 37 CFR 1.14. This collection is estimated to take 1.0 hour to complete, including gathering, preparing, and submitting the completed application form to the USPTO. Time will vary depending upon the individual case. Any comments on the amount of time you require to complete this form and/or suggestions for reducing this burden, should be sent to the Chief Information Officer, U.S. Patent and Trademark Office, U.S. Department of Commerce, P.O. Box 1450, Alexandria, VA 22313-1450. DO NOT SEND FEES OR COMPLETED FORMS TO THIS ADDRESS. SEND TO: Attention Certificate of Corrections Branch, Commissioner for Patents, P.O. Box 1450, Alexandria, VA 22313-1450.

If you need assistance in completing the form, call 1-800-PTO-9199 and selection option2.

Electronic Acknowledgement Receipt

EFS ID:	28577753
Application Number:	14067620
International Application Number:	
Confirmation Number:	4288
Title of Invention:	Nicking and Extension Amplification Reaction for the Exponential Amplification of Nucleic Acids
First Named Inventor/Applicant Name:	Brian K. Maples
Customer Number:	26161
Filer:	Ian J.S. Lodovice/Mary Florczak
Filer Authorized By:	Ian J.S. Lodovice
Attorney Docket Number:	30171-0025002 / ITI-001
Receipt Date:	09-MAR-2017
Filing Date:	30-OCT-2013
Time Stamp:	09:52:14
Application Type:	Utility under 35 USC 111(a)

Payment information:

Submitted with Payment	no

File Listing:

Document Number	Document Description	File Name	File Size(Bytes)/ Message Digest	Multi Part /.zip	Pages (if appl.)
1	Request for Certificate of Correction	30171RequestCertCor.pdf	130995 77006677bc04f750a03c6ecff3ac1a724b12450e	no	2

Warnings:

Information:	
Total Files Size (in bytes):	130995

This Acknowledgement Receipt evidences receipt on the noted date by the USPTO of the indicated documents, characterized by the applicant, and including page counts, where applicable. It serves as evidence of receipt similar to a Post Card, as described in MPEP 503.

New Applications Under 35 U.S.C. 111
If a new application is being filed and the application includes the necessary components for a filing date (see 37 CFR 1.53(b)-(d) and MPEP 506), a Filing Receipt (37 CFR 1.54) will be issued in due course and the date shown on this Acknowledgement Receipt will establish the filing date of the application.

National Stage of an International Application under 35 U.S.C. 371
If a timely submission to enter the national stage of an international application is compliant with the conditions of 35 U.S.C. 371 and other applicable requirements a Form PCT/DO/EO/903 indicating acceptance of the application as a national stage submission under 35 U.S.C. 371 will be issued in addition to the Filing Receipt, in due course.

New International Application Filed with the USPTO as a Receiving Office
If a new international application is being filed and the international application includes the necessary components for an international filing date (see PCT Article 11 and MPEP 1810), a Notification of the International Application Number and of the International Filing Date (Form PCT/RO/105) will be issued in due course, subject to prescriptions concerning national security, and the date shown on this Acknowledgement Receipt will establish the international filing date of the application.

UNITED STATES PATENT AND TRADEMARK OFFICE

UNITED STATES DEPARTMENT OF COMMERCE
United States Patent and Trademark Office
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P.O. Box 1450
Alexandria, Virginia 22313-1450
www.uspto.gov

APPLICATION NO.	ISSUE DATE	PATENT NO.	ATTORNEY DOCKET NO.	CONFIRMATION NO.
14/067,620	02/07/2017	9562263	30171-0025002 / ITI-001	4288

26161 7590 01/18/2017

FISH & RICHARDSON P.C. (BO)
P.O. BOX 1022
MINNEAPOLIS, MN 55440-1022

ISSUE NOTIFICATION

The projected patent number and issue date are specified above.

Determination of Patent Term Adjustment under 35 U.S.C. 154 (b)
(application filed on or after May 29, 2000)

The Patent Term Adjustment is 0 day(s). Any patent to issue from the above-identified application will include an indication of the adjustment on the front page.

If a Continued Prosecution Application (CPA) was filed in the above-identified application, the filing date that determines Patent Term Adjustment is the filing date of the most recent CPA.

Applicant will be able to obtain more detailed information by accessing the Patent Application Information Retrieval (PAIR) WEB site (http://pair.uspto.gov).

Any questions regarding the Patent Term Extension or Adjustment determination should be directed to the Office of Patent Legal Administration at (571)-272-7702. Questions relating to issue and publication fee payments should be directed to the Application Assistance Unit (AAU) of the Office of Data Management (ODM) at (571)-272-4200.

APPLICANT(s) (Please see PAIR WEB site http://pair.uspto.gov for additional applicants):

Ionian Technologies, Inc., San Diego, CA;
Brian K. Maples, Lake Forest, CA;
Rebecca C. Holmberg, San Diego, CA;
Andrew P. Miller, San Diego, CA;
Jarrod Provins, Dana Point, CA;
Richard Roth, Carlsbad, CA;
Jeffrey Mandell, San Diego, CA;

The United States represents the largest, most dynamic marketplace in the world and is an unparalleled location for business investment, innovation, and commercialization of new technologies. The USA offers tremendous resources and advantages for those who invest and manufacture goods here. Through SelectUSA, our nation works to encourage and facilitate business investment. To learn more about why the USA is the best country in the world to develop technology, manufacture products, and grow your business, visit SelectUSA.gov.

IR103 (Rev. 10/09)

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

First Named Inventor	:	Brian K. Maples	Art Unit	:	1637
Serial No.	:	14/067,620	Examiner	:	Angela Marie Bertagna
Filed	:	October 30, 2013	Confirmation No.	:	4288
			Notice of Allowance Date: December 15, 2016		
Title	:	NICKING AND EXTENSION AMPLIFICATION REACTION FOR THE EXPONENTIAL AMPLIFICATION OF NUCLEIC ACIDS			

MAIL STOP ISSUE FEE
Commissioner for Patents
P.O. Box 1450
Alexandria, VA 22313-1450

REPLY TO NOTICE OF ALLOWANCE

In response to the Notice of Allowance dated December 15, 2016, enclosed is a completed Part B - Fee(s) Transmittal.

The issue fee was previously paid on September 21, 2016 and no fee is believed to be due. However, please apply any necessary charges or credits to Deposit Account 06 1050, referencing the above attorney docket number.

Respectfully submitted,

Date: December 19, 2016

/Ian J.S. Lodovice, Reg. No. 59,749/

Ian J. Lodovice
Reg. No. 59,749

Customer Number 26161
Fish & Richardson P.C.
Telephone: (617) 956-5972
Facsimile: (877) 769-7945

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PART B - FEE(S) TRANSMITTAL

Complete and send this form, together with applicable fee(s), to: Mail **Mail Stop ISSUE FEE**
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Alexandria, Virginia 22313-1450
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CURRENT CORRESPONDENCE ADDRESS (Note: Use Block 1 for any change of address)

26161 7590 12/15/2016

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Note: A certificate of mailing can only be used for domestic mailings of the Fee(s) Transmittal. This certificate cannot be used for any other accompanying papers. Each additional paper, such as an assignment or formal drawing, must have its own certificate of mailing or transmission.

Certificate of Mailing or Transmission

I hereby certify that this Fee(s) Transmittal is being deposited with the United States Postal Service with sufficient postage for first class mail in an envelope addressed to the Mail Stop ISSUE FEE address above, or being facsimile transmitted to the USPTO (571) 273-2885, on the date indicated below.

(Depositor's name)

(Signature)

(Date)

APPLICATION NO.	FILING DATE	FIRST NAMED INVENTOR	ATTORNEY DOCKET NO.	CONFIRMATION NO.
14/067,620	10/30/2013	Brian K. Maples	30171-0025002	4288

TITLE OF INVENTION: NICKING AND EXTENSION AMPLIFICATION REACTION FOR THE EXPONENTIAL AMPLIFICATION OF NUCLEIC ACIDS

APPLN. TYPE	ENTITY STATUS	ISSUE FEE DUE	PUBLICATION FEE DUE	PREV. PAID ISSUE FEE	TOTAL FEE(S) DUE	DATE DUE
nonprovisional	LARGE	$0	$0	$960.00	$0	03/15/2017

EXAMINER	ART UNIT	CLASS-SUBCLASS
BERTAGNA, ANGELA MARIE	1637	435-006120

1. Change of correspondence address or indication of "Fee Address" (37 CFR 1.363).

[ ] Change of correspondence address (or Change of Correspondence Address form PTO/SB/122) attached.

[ ] "Fee Address" indication (or "Fee Address" Indication form PTO/SB/47; Rev 03-02 or more recent) attached. **Use of a Customer Number is required.**

2. For printing on the patent front page, list

(1) the names of up to 3 registered patent attorneys or agents OR, alternatively,

(2) the name of a single firm (having as a member a registered attorney or agent) and the names of up to 2 registered patent attorneys or agents. If no name is listed, no name will be printed.

1 Fish & Richardson P.C.
2
3

3. ASSIGNEE NAME AND RESIDENCE DATA TO BE PRINTED ON THE PATENT (print or type)

PLEASE NOTE: Unless an assignee is identified below, no assignee data will appear on the patent. If an assignee is identified below, the document has been filed for recordation as set forth in 37 CFR 3.11. Completion of this form is NOT a substitute for filing an assignment.

(A) NAME OF ASSIGNEE
Ionian Technologies, Inc.

(B) RESIDENCE: (CITY and STATE OR COUNTRY)
San Diego, CA

Please check the appropriate assignee category or categories (will not be printed on the patent): [ ] Individual [X] Corporation or other private group entity [ ] Government

4a. The following fee(s) are submitted:

[X] Issue Fee
[ ] Publication Fee (No small entity discount permitted)
[ ] Advance Order - # of Copies ______

4b. Payment of Fee(s): **(Please first reapply any previously paid issue fee shown above)**

[ ] A check in the amount of the fee(s) is enclosed.
[ ] Payment by credit card. Form PTO-2038 is attached.
[X] The Director is hereby authorized to charge the required fee(s), or credit any overpayment, to Deposit Account Number 06-1050

5. **Change in Entity Status** (from status indicated above)

[ ] Applicant certifying micro entity status. See 37 CFR 1.29 — **NOTE**: Absent a valid certification of Micro Entity Status (see form PTO/SB/15A and 15B), issue fee payment in the micro entity amount will not be accepted at the risk of application abandonment.

[ ] Applicant asserting small entity status. See 37 CFR 1.27. — **NOTE**: If the application was previously under micro entity status, checking this box will be taken to be a notification of loss of entitlement to micro entity status.

[ ] Applicant changing to regular undiscounted fee status. — **NOTE**: Checking this box will be taken to be a notification of loss of entitlement to small or micro entity status, as applicable.

The Director of the USPTO is requested to apply the Issue Fee and Publication Fee (if any) or to re-apply any previously paid issue fee to the application identified above.
NOTE: The Issue Fee and Publication Fee (if required) will not be accepted from anyone other than the applicant; a registered attorney or agent; or the assignee or other party in interest as shown by the records of the United States Patent and Trademark Office.

Authorized Signature /Ian J.S. Lodovice, Reg. No. 59,749/ Date December 19, 2016

Typed or printed name Ian J. Lodovice Registration No. 59,749

23598864.doc

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

First Named Inventor	:	Brian K. Maples	Art Unit	:	1637
Serial No.	:	14/067,620	Examiner	:	Angela Marie Bertagna
Filed	:	October 30, 2013	Conf. No.	:	4288
Title	:	NICKING AND EXTENSION AMPLIFICATION REACTION FOR THE EXPONENTIAL AMPLIFICATION OF NUCLEIC ACIDS			

Mail Stop Amendment
Commissioner for Patents
P.O. Box 1450
Alexandria, VA 22313-1450

INTERVIEW SUMMARY

The undersigned representative thanks the Examiner for the courtesy of the telephonic interview held on November 29, 2016.

The substance of the interview included a discussion of claims 67, 95, and 107 and the reference Nelson et al. During the interview, an agreement was reached on December 1, 2016. Applicant acknowledges and thanks Examiner Bertagna for the Examiner-Initiated Interview Summary dated December 15, 2016.

Respectfully submitted,

Date: December 19, 2016

/Ian J.S. Lodovice, Reg. No. 59,749/
Ian J. Lodovice
Reg. No. 59,749

Customer Number 26161
Fish & Richardson P.C.
Telephone: (617) 956-5972
Facsimile: (877) 769-7945
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Electronic Acknowledgement Receipt

EFS ID:	27831554
Application Number:	14067620
International Application Number:	
Confirmation Number:	4288
Title of Invention:	Nicking and Extension Amplification Reaction for the Exponential Amplification of Nucleic Acids
First Named Inventor/Applicant Name:	Brian K. Maples
Customer Number:	26161
Filer:	Ian J.S. Lodovice/Stacey Hill
Filer Authorized By:	Ian J.S. Lodovice
Attorney Docket Number:	30171-0025002 / ITI-001
Receipt Date:	19-DEC-2016
Filing Date:	30-OCT-2013
Time Stamp:	14:44:33
Application Type:	Utility under 35 USC 111(a)

Payment information:

Submitted with Payment	no

File Listing:

Document Number	Document Description	File Name	File Size(Bytes)/ Message Digest	Multi Part /.zip	Pages (if appl.)
1	Issue Fee Payment (PTO-85B)	30171IssueFee.pdf	196349 9d5b64259f9b792d89ffb240090337b27c668509	no	2

Warnings:

Information:					
2	Applicant summary of interview with examiner	301710025002IS.pdf	71354 e5d6e1ee15310983a2c328fcb1788d623de38897	no	1
Warnings:					
Information:					
		Total Files Size (in bytes):	267703		

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New Applications Under 35 U.S.C. 111
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TKW

PART B - FEE(S) TRANSMITTAL

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CURRENT CORRESPONDENCE ADDRESS (Note: Use Block 1 for any change of address)

26161 7590 12/15/2016

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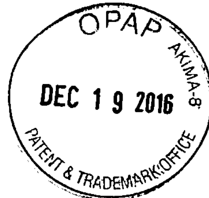

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(Depositor's name)

(Signature)

(Date)

APPLICATION NO.	FILING DATE	FIRST NAMED INVENTOR	ATTORNEY DOCKET NO.	CONFIRMATION NO.
14/067,620	10/30/2013	Brian K. Maples	30171-0025002	4288

TITLE OF INVENTION: NICKING AND EXTENSION AMPLIFICATION REACTION FOR THE EXPONENTIAL AMPLIFICATION OF NUCLEIC ACIDS

APPLN. TYPE	ENTITY STATUS	ISSUE FEE DUE	PUBLICATION FEE DUE	PREV. PAID ISSUE FEE	TOTAL FEE(S) DUE	DATE DUE
nonprovisional	LARGE	$0	$0	$960.00	$0	03/15/2017

EXAMINER	ART UNIT	CLASS-SUBCLASS
BERTAGNA, ANGELA MARIE	1637	435-006120

1. Change of correspondence address or indication of "Fee Address" (37 CFR 1.363).

[ ] Change of correspondence address (or Change of Correspondence Address form PTO/SB/122) attached.

[ ] "Fee Address" indication (or "Fee Address" Indication form PTO/SB/47; Rev 03-02 or more recent) attached. **Use of a Customer Number is required.**

2. For printing on the patent front page, list

(1) the names of up to 3 registered patent attorneys or agents OR, alternatively,

(2) the name of a single firm (having as a member a registered attorney or agent) and the names of up to 2 registered patent attorneys or agents. If no name is listed, no name will be printed.

1 Fish & Richardson P.C.
2 ______
3 ______

3. ASSIGNEE NAME AND RESIDENCE DATA TO BE PRINTED ON THE PATENT (print or type)

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(A) NAME OF ASSIGNEE
Ionian Technologies, Inc.

(B) RESIDENCE: (CITY and STATE OR COUNTRY)
San Diego, CA

Please check the appropriate assignee category or categories (will not be printed on the patent): [ ] Individual [X] Corporation or other private group entity [ ] Government

4a. The following fee(s) are submitted:

[X] Issue Fee
[ ] Publication Fee (No small entity discount permitted)
[ ] Advance Order - # of Copies ______

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[ ] Payment by credit card. Form PTO-2038 is attached.
[X] The Director is hereby authorized to charge the required fee(s), or credit any overpayment, to Deposit Account Number 06-1050

5. **Change in Entity Status** (from status indicated above)

[ ] Applicant certifying micro entity status. See 37 CFR 1.29 — **NOTE**: Absent a valid certification of Micro Entity Status (see form PTO/SB/15A and 15B), issue fee payment in the micro entity amount will not be accepted at the risk of application abandonment.

[ ] Applicant asserting small entity status. See 37 CFR 1.27. — **NOTE**: If the application was previously under micro entity status, checking this box will be taken to be a notification of loss of entitlement to micro entity status.

[ ] Applicant changing to regular undiscounted fee status. — **NOTE**: Checking this box will be taken to be a notification of loss of entitlement to small or micro entity status, as applicable.

The Director of the USPTO is requested to apply the Issue Fee and Publication Fee (if any) or to re-apply any previously paid issue fee to the application identified above.
NOTE: The Issue Fee and Publication Fee (if required) will not be accepted from anyone other than the applicant; a registered attorney or agent; or the assignee or other party in interest as shown by the records of the United States Patent and Trademark Office.

Authorized Signature /Ian J.S. Lodovice, Reg. No. 59,749/ Date December 19, 2016

Typed or printed name Ian J. Lodovice Registration No. 59,749

23598864.doc

Adjustment date: 12/21/2016 SDIRETA2
09/22/2016 INTEFSW 00005299 061050 14067620
01 FC:1501 960.00 CR

12/21/2016 SDIRETA2 00000003 061050 14067620
01 FC:1501 960.00 DA

UNITED STATES PATENT AND TRADEMARK OFFICE

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United States Patent and Trademark Office
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Alexandria, Virginia 22313-1450
www.uspto.gov

NOTICE OF ALLOWANCE AND FEE(S) DUE

26161 7590 12/15/2016

FISH & RICHARDSON P.C. (BO)
P.O. BOX 1022
MINNEAPOLIS, MN 55440-1022

EXAMINER	
BERTAGNA, ANGELA MARIE	
ART UNIT	PAPER NUMBER
1637	

DATE MAILED: 12/15/2016

APPLICATION NO.	FILING DATE	FIRST NAMED INVENTOR	ATTORNEY DOCKET NO.	CONFIRMATION NO.
14/067,620	10/30/2013	Brian K. Maples	30171-0025002 / ITI-001	4288

TITLE OF INVENTION: Nicking and Extension Amplification Reaction for the Exponential Amplification of Nucleic Acids

APPLN. TYPE	ENTITY STATUS	ISSUE FEE DUE	PUBLICATION FEE DUE	PREV. PAID ISSUE FEE	TOTAL FEE(S) DUE	DATE DUE
nonprovisional	UNDISCOUNTED	$960	$0	$960	$960	03/15/2017

THE APPLICATION IDENTIFIED ABOVE HAS BEEN EXAMINED AND IS ALLOWED FOR ISSUANCE AS A PATENT. PROSECUTION ON THE MERITS IS CLOSED. THIS NOTICE OF ALLOWANCE IS NOT A GRANT OF PATENT RIGHTS. THIS APPLICATION IS SUBJECT TO WITHDRAWAL FROM ISSUE AT THE INITIATIVE OF THE OFFICE OR UPON PETITION BY THE APPLICANT. SEE 37 CFR 1.313 AND MPEP 1308.

THE ISSUE FEE AND PUBLICATION FEE (IF REQUIRED) MUST BE PAID WITHIN THREE MONTHS FROM THE MAILING DATE OF THIS NOTICE OR THIS APPLICATION SHALL BE REGARDED AS ABANDONED. THIS STATUTORY PERIOD CANNOT BE EXTENDED. SEE 35 U.S.C. 151. THE ISSUE FEE DUE INDICATED ABOVE DOES NOT REFLECT A CREDIT FOR ANY PREVIOUSLY PAID ISSUE FEE IN THIS APPLICATION. IF AN ISSUE FEE HAS PREVIOUSLY BEEN PAID IN THIS APPLICATION (AS SHOWN ABOVE), THE RETURN OF PART B OF THIS FORM WILL BE CONSIDERED A REQUEST TO REAPPLY THE PREVIOUSLY PAID ISSUE FEE TOWARD THE ISSUE FEE NOW DUE.

HOW TO REPLY TO THIS NOTICE:

I. Review the ENTITY STATUS shown above. If the ENTITY STATUS is shown as SMALL or MICRO, verify whether entitlement to that entity status still applies.

If the ENTITY STATUS is the same as shown above, pay the TOTAL FEE(S) DUE shown above.

If the ENTITY STATUS is changed from that shown above, on PART B - FEE(S) TRANSMITTAL, complete section number 5 titled "Change in Entity Status (from status indicated above)".

For purposes of this notice, small entity fees are 1/2 the amount of undiscounted fees, and micro entity fees are 1/2 the amount of small entity fees.

II. PART B - FEE(S) TRANSMITTAL, or its equivalent, must be completed and returned to the United States Patent and Trademark Office (USPTO) with your ISSUE FEE and PUBLICATION FEE (if required). If you are charging the fee(s) to your deposit account, section "4b" of Part B - Fee(s) Transmittal should be completed and an extra copy of the form should be submitted. If an equivalent of Part B is filed, a request to reapply a previously paid issue fee must be clearly made, and delays in processing may occur due to the difficulty in recognizing the paper as an equivalent of Part B.

III. All communications regarding this application must give the application number. Please direct all communications prior to issuance to Mail Stop ISSUE FEE unless advised to the contrary.

IMPORTANT REMINDER: Utility patents issuing on applications filed on or after Dec. 12, 1980 may require payment of maintenance fees. It is patentee's responsibility to ensure timely payment of maintenance fees when due.

PART B - FEE(S) TRANSMITTAL

Complete and send this form, together with applicable fee(s), to: Mail **Mail Stop ISSUE FEE**
Commissioner for Patents
P.O. Box 1450
Alexandria, Virginia 22313-1450
or Fax (571)-273-2885

INSTRUCTIONS: This form should be used for transmitting the ISSUE FEE and PUBLICATION FEE (if required). Blocks 1 through 5 should be completed where appropriate. All further correspondence including the Patent, advance orders and notification of maintenance fees will be mailed to the current correspondence address as indicated unless corrected below or directed otherwise in Block 1, by (a) specifying a new correspondence address; and/or (b) indicating a separate "FEE ADDRESS" for maintenance fee notifications.

CURRENT CORRESPONDENCE ADDRESS (Note: Use Block 1 for any change of address)

26161 7590 12/15/2016

FISH & RICHARDSON P.C. (BO)
P.O. BOX 1022
MINNEAPOLIS, MN 55440-1022

Note: A certificate of mailing can only be used for domestic mailings of the Fee(s) Transmittal. This certificate cannot be used for any other accompanying papers. Each additional paper, such as an assignment or formal drawing, must have its own certificate of mailing or transmission.

Certificate of Mailing or Transmission

I hereby certify that this Fee(s) Transmittal is being deposited with the United States Postal Service with sufficient postage for first class mail in an envelope addressed to the Mail Stop ISSUE FEE address above, or being facsimile transmitted to the USPTO (571) 273-2885, on the date indicated below.

(Depositor's name)
(Signature)
(Date)

APPLICATION NO.	FILING DATE	FIRST NAMED INVENTOR	ATTORNEY DOCKET NO.	CONFIRMATION NO.
14/067,620	10/30/2013	Brian K. Maples	30171-0025002 / ITI-001	4288

TITLE OF INVENTION: Nicking and Extension Amplification Reaction for the Exponential Amplification of Nucleic Acids

APPLN. TYPE	ENTITY STATUS	ISSUE FEE DUE	PUBLICATION FEE DUE	PREV. PAID ISSUE FEE	TOTAL FEE(S) DUE	DATE DUE
nonprovisional	UNDISCOUNTED	$960	$0	$960	$960	03/15/2017

EXAMINER	ART UNIT	CLASS-SUBCLASS
BERTAGNA, ANGELA MARIE	1637	435-006120

1. Change of correspondence address or indication of "Fee Address" (37 CFR 1.363).
   - ❑ Change of correspondence address (or Change of Correspondence Address form PTO/SB/122) attached.
   - ❑ "Fee Address" indication (or "Fee Address" Indication form PTO/SB/47; Rev 03-02 or more recent) attached. **Use of a Customer Number is required.**

2. For printing on the patent front page, list
   (1) The names of up to 3 registered patent attorneys or agents OR, alternatively,
   (2) The name of a single firm (having as a member a registered attorney or agent) and the names of up to 2 registered patent attorneys or agents. If no name is listed, no name will be printed.

   1 ____________________
   2 ____________________
   3 ____________________

3. ASSIGNEE NAME AND RESIDENCE DATA TO BE PRINTED ON THE PATENT (print or type)

   PLEASE NOTE: Unless an assignee is identified below, no assignee data will appear on the patent. If an assignee is identified below, the document has been filed for recordation as set forth in 37 CFR 3.11. Completion of this form is NOT a substitute for filing an assignment.

   (A) NAME OF ASSIGNEE

   (B) RESIDENCE: (CITY and STATE OR COUNTRY)

Please check the appropriate assignee category or categories (will not be printed on the patent) : ❑ Individual ❑ Corporation or other private group entity ❑ Government

4a. The following fee(s) are submitted:
- ❑ Issue Fee
- ❑ Publication Fee (No small entity discount permitted)
- ❑ Advance Order - # of Copies ____________

4b. Payment of Fee(s): (**Please first reapply any previously paid issue fee shown above**)
- ❑ A check is enclosed.
- ❑ Payment by credit card. Form PTO-2038 is attached.
- ❑ The director is hereby authorized to charge the required fee(s), any deficiency, or credits any overpayment, to Deposit Account Number __________ (enclose an extra copy of this form).

5. **Change in Entity Status** (from status indicated above)
- ❑ Applicant certifying micro entity status. See 37 CFR 1.29 — NOTE: Absent a valid certification of Micro Entity Status (see forms PTO/SB/15A and 15B), issue fee payment in the micro entity amount will not be accepted at the risk of application abandonment.
- ❑ Applicant asserting small entity status. See 37 CFR 1.27 — NOTE: If the application was previously under micro entity status, checking this box will be taken to be a notification of loss of entitlement to micro entity status.
- ❑ Applicant changing to regular undiscounted fee status. — NOTE: Checking this box will be taken to be a notification of loss of entitlement to small or micro entity status, as applicable.

NOTE: This form must be signed in accordance with 37 CFR 1.31 and 1.33. See 37 CFR 1.4 for signature requirements and certifications.

Authorized Signature ____________________ Date ____________________

Typed or printed name ____________________ Registration No. ____________________

PTOL-85 Part B (10-13) Approved for use through 10/31/2013. OMB 0651-0033 U.S. Patent and Trademark Office; U.S. DEPARTMENT OF COMMERCE

UNITED STATES PATENT AND TRADEMARK OFFICE

UNITED STATES DEPARTMENT OF COMMERCE
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APPLICATION NO.	FILING DATE	FIRST NAMED INVENTOR	ATTORNEY DOCKET NO.	CONFIRMATION NO.
14/067,620	10/30/2013	Brian K. Maples	30171-0025002 / ITI-001	4288

26161 7590 12/15/2016
FISH & RICHARDSON P.C. (BO)
P.O. BOX 1022
MINNEAPOLIS, MN 55440-1022

EXAMINER	
BERTAGNA, ANGELA MARIE	

ART UNIT	PAPER NUMBER
1637	

DATE MAILED: 12/15/2016

Determination of Patent Term Adjustment under 35 U.S.C. 154 (b)
(Applications filed on or after May 29, 2000)

The Office has discontinued providing a Patent Term Adjustment (PTA) calculation with the Notice of Allowance.

Section 1(h)(2) of the AIA Technical Corrections Act amended 35 U.S.C. 154(b)(3)(B)(i) to eliminate the requirement that the Office provide a patent term adjustment determination with the notice of allowance. See Revisions to Patent Term Adjustment, 78 Fed. Reg. 19416, 19417 (Apr. 1, 2013). Therefore, the Office is no longer providing an initial patent term adjustment determination with the notice of allowance. The Office will continue to provide a patent term adjustment determination with the Issue Notification Letter that is mailed to applicant approximately three weeks prior to the issue date of the patent, and will include the patent term adjustment on the patent. Any request for reconsideration of the patent term adjustment determination (or reinstatement of patent term adjustment) should follow the process outlined in 37 CFR 1.705.

Any questions regarding the Patent Term Extension or Adjustment determination should be directed to the Office of Patent Legal Administration at (571)-272-7702. Questions relating to issue and publication fee payments should be directed to the Customer Service Center of the Office of Patent Publication at 1-(888)-786-0101 or (571)-272-4200.

PTOL-85 (Rev. 02/11)

OMB Clearance and PRA Burden Statement for PTOL-85 Part B

The Paperwork Reduction Act (PRA) of 1995 requires Federal agencies to obtain Office of Management and Budget approval before requesting most types of information from the public. When OMB approves an agency request to collect information from the public, OMB (i) provides a valid OMB Control Number and expiration date for the agency to display on the instrument that will be used to collect the information and (ii) requires the agency to inform the public about the OMB Control Number's legal significance in accordance with 5 CFR 1320.5(b).

The information collected by PTOL-85 Part B is required by 37 CFR 1.311. The information is required to obtain or retain a benefit by the public which is to file (and by the USPTO to process) an application. Confidentiality is governed by 35 U.S.C. 122 and 37 CFR 1.14. This collection is estimated to take 12 minutes to complete, including gathering, preparing, and submitting the completed application form to the USPTO. Time will vary depending upon the individual case. Any comments on the amount of time you require to complete this form and/or suggestions for reducing this burden, should be sent to the Chief Information Officer, U.S. Patent and Trademark Office, U.S. Department of Commerce, P.O. Box 1450, Alexandria, Virginia 22313-1450. DO NOT SEND FEES OR COMPLETED FORMS TO THIS ADDRESS. SEND TO: Commissioner for Patents, P.O. Box 1450, Alexandria, Virginia 22313-1450. Under the Paperwork Reduction Act of 1995, no persons are required to respond to a collection of information unless it displays a valid OMB control number.

Privacy Act Statement

The Privacy Act of 1974 (P.L. 93-579) requires that you be given certain information in connection with your submission of the attached form related to a patent application or patent. Accordingly, pursuant to the requirements of the Act, please be advised that: (1) the general authority for the collection of this information is 35 U.S.C. 2(b)(2); (2) furnishing of the information solicited is voluntary; and (3) the principal purpose for which the information is used by the U.S. Patent and Trademark Office is to process and/or examine your submission related to a patent application or patent. If you do not furnish the requested information, the U.S. Patent and Trademark Office may not be able to process and/or examine your submission, which may result in termination of proceedings or abandonment of the application or expiration of the patent.

The information provided by you in this form will be subject to the following routine uses:

1. The information on this form will be treated confidentially to the extent allowed under the Freedom of Information Act (5 U.S.C. 552) and the Privacy Act (5 U.S.C 552a). Records from this system of records may be disclosed to the Department of Justice to determine whether disclosure of these records is required by the Freedom of Information Act.
2. A record from this system of records may be disclosed, as a routine use, in the course of presenting evidence to a court, magistrate, or administrative tribunal, including disclosures to opposing counsel in the course of settlement negotiations.
3. A record in this system of records may be disclosed, as a routine use, to a Member of Congress submitting a request involving an individual, to whom the record pertains, when the individual has requested assistance from the Member with respect to the subject matter of the record.
4. A record in this system of records may be disclosed, as a routine use, to a contractor of the Agency having need for the information in order to perform a contract. Recipients of information shall be required to comply with the requirements of the Privacy Act of 1974, as amended, pursuant to 5 U.S.C. 552a(m).
5. A record related to an International Application filed under the Patent Cooperation Treaty in this system of records may be disclosed, as a routine use, to the International Bureau of the World Intellectual Property Organization, pursuant to the Patent Cooperation Treaty.
6. A record in this system of records may be disclosed, as a routine use, to another federal agency for purposes of National Security review (35 U.S.C. 181) and for review pursuant to the Atomic Energy Act (42 U.S.C. 218(c)).
7. A record from this system of records may be disclosed, as a routine use, to the Administrator, General Services, or his/her designee, during an inspection of records conducted by GSA as part of that agency's responsibility to recommend improvements in records management practices and programs, under authority of 44 U.S.C. 2904 and 2906. Such disclosure shall be made in accordance with the GSA regulations governing inspection of records for this purpose, and any other relevant (i.e., GSA or Commerce) directive. Such disclosure shall not be used to make determinations about individuals.
8. A record from this system of records may be disclosed, as a routine use, to the public after either publication of the application pursuant to 35 U.S.C. 122(b) or issuance of a patent pursuant to 35 U.S.C. 151. Further, a record may be disclosed, subject to the limitations of 37 CFR 1.14, as a routine use, to the public if the record was filed in an application which became abandoned or in which the proceedings were terminated and which application is referenced by either a published application, an application open to public inspection or an issued patent.
9. A record from this system of records may be disclosed, as a routine use, to a Federal, State, or local law enforcement agency, if the USPTO becomes aware of a violation or potential violation of law or regulation.

Notice of Allowability	Application No. 14/067,620	Applicant(s) MAPLES ET AL.	
	Examiner Angela M. Bertagna	Art Unit 1637	AIA (First Inventor to File) Status No

-- The MAILING DATE of this communication appears on the cover sheet with the correspondence address--

All claims being allowable, PROSECUTION ON THE MERITS IS (OR REMAINS) CLOSED in this application. If not included herewith (or previously mailed), a Notice of Allowance (PTOL-85) or other appropriate communication will be mailed in due course. **THIS NOTICE OF ALLOWABILITY IS NOT A GRANT OF PATENT RIGHTS.** This application is subject to withdrawal from issue at the initiative of the Office or upon petition by the applicant. See 37 CFR 1.313 and MPEP 1308.

1. ☒ This communication is responsive to *the RCE filed on 11/1/16 and the interview of 11/29/16*.
   ☐ A declaration(s)/affidavit(s) under **37 CFR 1.130(b)** was/were filed on______.
2. ☐ An election was made by the applicant in response to a restriction requirement set forth during the interview on _____; the restriction requirement and election have been incorporated into this action.
3. ☒ The allowed claim(s) is/are *67-83,85,86,88,90,92 and 95-107*. As a result of the allowed claim(s), you may be eligible to benefit from the **Patent Prosecution Highway** program at a participating intellectual property office for the corresponding application. For more information, please see http://www.uspto.gov/patents/init_events/pph/index.jsp or send an inquiry to PPHfeedback@uspto.gov.
4. ☐ Acknowledgment is made of a claim for foreign priority under 35 U.S.C. § 119(a)-(d) or (f).
   Certified copies:
   a) ☐ All b) ☐ Some *c) ☐ None of the:
   1. ☐ Certified copies of the priority documents have been received.
   2. ☐ Certified copies of the priority documents have been received in Application No. _____ .
   3. ☐ Copies of the certified copies of the priority documents have been received in this national stage application from the International Bureau (PCT Rule 17.2(a)).
   * Certified copies not received: _____.

Applicant has THREE MONTHS FROM THE "MAILING DATE" of this communication to file a reply complying with the requirements noted below. Failure to timely comply will result in ABANDONMENT of this application.
THIS THREE-MONTH PERIOD IS NOT EXTENDABLE.

5. ☐ CORRECTED DRAWINGS (as "replacement sheets") must be submitted.
   ☐ including changes required by the attached Examiner's Amendment / Comment or in the Office action of Paper No./Mail Date _____.
   Identifying indicia such as the application number (see 37 CFR 1.84(c)) should be written on the drawings in the front (not the back) of each sheet. Replacement sheet(s) should be labeled as such in the header according to 37 CFR 1.121(d).
6. ☐ DEPOSIT OF and/or INFORMATION about the deposit of BIOLOGICAL MATERIAL must be submitted. Note the attached Examiner's comment regarding REQUIREMENT FOR THE DEPOSIT OF BIOLOGICAL MATERIAL.

Attachment(s)
1. ☐ Notice of References Cited (PTO-892)
2. ☒ Information Disclosure Statements (PTO/SB/08), Paper No./Mail Date _____
3. ☐ Examiner's Comment Regarding Requirement for Deposit of Biological Material
4. ☒ Interview Summary (PTO-413), Paper No./Mail Date _____ .
5. ☒ Examiner's Amendment/Comment
6. ☐ Examiner's Statement of Reasons for Allowance
7. ☐ Other _____.

/Angela M. Bertagna/
Primary Examiner, Art Unit 1637

U.S. Patent and Trademark Office
PTOL-37 (Rev. 08-13) **Notice of Allowability** Part of Paper No./Mail Date 20161122

DETAILED ACTION

Notice of Pre-AIA or AIA Status

1. The present application is being examined under the pre-AIA first to invent provisions.

Continued Examination Under 37 CFR 1.114

2. A request for continued examination under 37 CFR 1.114, including the fee set forth in 37 CFR 1.17€, was filed in this application after allowance or after an Office action under *Ex Parte Quayle*, 25 USPQ 74, 453 O.G. 213 (Comm'r Pat. 1935). Since this application is eligible for continued examination under 37 CFR 1.114, and the fee set forth in 37 CFR 1.17€ has been timely paid, prosecution in this application has been reopened pursuant to 37 CFR 1.114. Applicant's submission filed on November 1, 2016 has been entered.

Information Disclosure Statement

3. The Information Disclosure Statement filed on November 1, 2016 has been considered.

EXAMINER'S AMENDMENT

4. An examiner's amendment to the record appears below. Should the changes and/or additions be unacceptable to applicant, an amendment may be filed as provided by 37 CFR 1.312. To ensure consideration of such an amendment, it MUST be submitted no later than the payment of the issue fee.

Authorization for this examiner's amendment was given in an interview with Ian Lodovice (Reg. No. 59,749) on December 1, 2016.

The application has been amended as follows:

In the claims:

In **claim 67, lines 14-15**, please delete the words “(iii) a first oligonucleotide comprising a nicking site and a nicking enzyme binding site, and” and insert --(iii) a first oligonucleotide comprising a 5’ portion that comprises a nicking enzyme binding site that is non-complementary to the target polynucleotide sequence and a 3’ portion that hybridizes to the target polynucleotide sequence, and-- therefor.

In **claim 67, lines 16-17**, please delete the words “(iv) a second oligonucleotide comprising a nicking site and a nicking enzyme binding site,” and insert --(iv) a second oligonucleotide comprising a 5’ portion that comprises a nicking enzyme binding site that is non-complementary to the target polynucleotide sequence and a 3’ portion that hybridizes to the target polynucleotide sequence,-- therefor.

In **claim 95, lines 15-16**, please delete the words “(iii) a first oligonucleotide comprising a nicking site and a nicking enzyme binding site, and” and insert --(iii) a first oligonucleotide comprising a 5’ portion that comprises a nicking enzyme binding site that is non-complementary to the target polynucleotide sequence and a 3’ portion that hybridizes to the target polynucleotide sequence, and-- therefor.

In **claim 95, lines 17-18**, please delete the words “(iv) a second oligonucleotide comprising a nicking site and a nicking enzyme binding site” and insert --(iv) a second oligonucleotide comprising a 5’ portion that comprises a nicking enzyme binding site that is non-complementary to the target polynucleotide sequence and a 3’ portion that hybridizes to the target polynucleotide sequence-- therefor.

In **claim 107, lines 14-15**, please delete the words "(iii) a first oligonucleotide comprising a nicking site and a nicking enzyme binding site, and" and insert --(iii) a first oligonucleotide comprising a 5' portion that comprises a nicking enzyme binding site that is non-complementary to the target polynucleotide sequence and a 3' portion that hybridizes to the target polynucleotide sequence, and-- therefor.

In **claim 107, lines 16-17**, please delete the words "(iv) a second oligonucleotide comprising a nicking site and a nicking enzyme binding site" and insert --(iv) a second oligonucleotide comprising a 5' portion that comprises a nicking enzyme binding site that is non-complementary to the target polynucleotide sequence and a 3' portion that hybridizes to the target polynucleotide sequence,-- therefor.

Allowable Subject Matter

5. Claims 67-83, 85, 86, 88, 90, 92, and 95-107 are allowed.

Conclusion

6. Any inquiry concerning this communication or earlier communications from the examiner should be directed to Angela M. Bertagna whose telephone number is (571)272-8291. The examiner can normally be reached on Monday-Friday, 9-5.

If attempts to reach the examiner by telephone are unsuccessful, the examiner's supervisor, Gary Benzion can be reached on (571)272-0782. The fax phone number for the organization where this application or proceeding is assigned is 571-273-8300.

Information regarding the status of an application may be obtained from the Patent Application Information Retrieval (PAIR) system. Status information for published applications may be obtained from either Private PAIR or Public PAIR. Status information for unpublished applications is available through Private PAIR only. For more information about the PAIR system, see http://pair-direct.uspto.gov. Should you have questions on access to the Private PAIR system, contact the Electronic Business Center (EBC) at 866-217-9197 (toll-free). If you would like assistance from a USPTO Customer Service Representative or access to the automated information system, call 800-786-9199 (IN USA OR CANADA) or 571-272-1000.

/Angela M. Bertagna/
Primary Examiner, Art Unit 1637

Examiner-Initiated Interview Summary	**Application No.** 14/067,620	**Applicant(s)** MAPLES ET AL.	
	Examiner Angela M. Bertagna	**Art Unit** 1637	

All participants (applicant, applicant's representative, PTO personnel):

(1) *Angela M. Bertagna (PTO)*.

(2) *Ian Lodovice (Applicant's representative)*.

(3) *Belinda Lew (Applicant's representative)*.

(4)_____.

Date of Interview: *29 November 2016*.

Type: ☒ Telephonic ☐ Video Conference
☐ Personal [copy given to: ☐ applicant ☐ applicant's representative]

Exhibit shown or demonstration conducted: ☐ Yes ☒ No.
If Yes, brief description: _____.

Issues Discussed ☐101 ☐112 ☒102 ☒103 ☐Others
(For each of the checked box(es) above, please describe below the issue and detailed description of the discussion)

Claim(s) discussed: *67,95 and 107*.

Identification of prior art discussed: *Nelson et al. (US 2009/0011472 A1), which is cited on the IDS filed on 11/1/16*.

Substance of Interview
(For each issue discussed, provide a detailed description and indicate if agreement was reached. Some topics may include: identification or clarification of a reference or a portion thereof, claim interpretation, proposed amendments, arguments of any applied references etc...)

We discussed amendments to claims 67, 95, and 107 to distinguish them from the Nelson reference. Agreement was reached on 12/1/16.

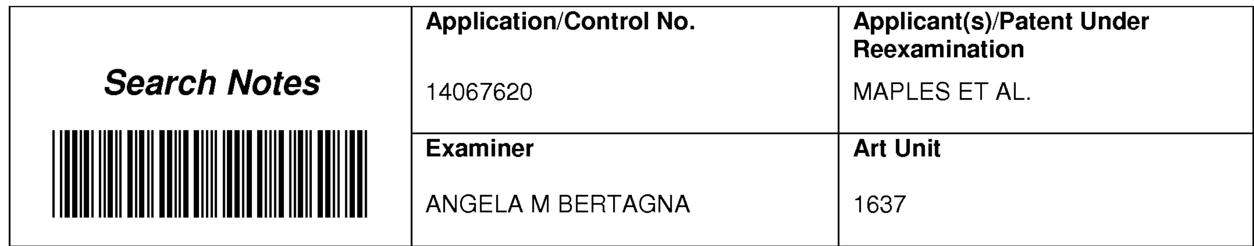
Applicant recordation instructions: It is not necessary for applicant to provide a separate record of the substance of interview.

Examiner recordation instructions: Examiners must summarize the substance of any interview of record. A complete and proper recordation of the substance of an interview should include the items listed in MPEP 713.04 for complete and proper recordation including the identification of the general thrust of each argument or issue discussed, a general indication of any other pertinent matters discussed regarding patentability and the general results or outcome of the interview, to include an indication as to whether or not agreement was reached on the issues raised.

☐ Attachment

/Angela M. Bertagna/ Primary Examiner, Art Unit 1637	

Search Notes	Application/Control No.	Applicant(s)/Patent Under Reexamination
	14067620	MAPLES ET AL.
	Examiner	**Art Unit**
	ANGELA M BERTAGNA	1637



CPC- SEARCHED

Symbol	Date	Examiner

CPC COMBINATION SETS - SEARCHED

Symbol	Date	Examiner

US CLASSIFICATION SEARCHED

Class	Subclass	Date	Examiner

SEARCH NOTES

Search Notes	Date	Examiner
searched all inventors by name	10/10/2014	amb
EAST search history attached	10/10/2014; 10/14/2014	amb
Google Scholar (search terms included "nicking", "isothermal", and "RNA")	10/10/2014; 10/14/2014	amb
reviewed related cases - 14067623 & 11778018	10/10/2014	amb
updated search (text & inventor name)	2/18/15; 2/19/15	amb
updated search (text & inventor name)	2/4/2016	amb
updated search (text & inventor name)	8/24/2016; 8/26/2016; 9/8/2016	amb
updated search (text & inventor name)	11/22/16-11/23/16; 12/7/16	amb

INTERFERENCE SEARCH

US Class/ CPC Symbol	US Subclass / CPC Group	Date	Examiner
searched USPGPUBS (text & inventor name)		9/8/2016; 12/7/16	amb

Receipt date: 11/01/2016

14067620 - GAU: 1637

Substitute Disclosure Form	U.S. Department of Commerce Patent and Trademark Office	Attorney Docket No. 30171-0025002	Application No. 14/067,620
Information Disclosure Statement by Applicant (Use several sheets if necessary) (37 CFR §1.98(b))		Applicant Ionian Technologies Inc.	
		Filing Date October 30, 2013	Group Art Unit 1637

U.S. Patent Documents

Examiner Initial	Desig. ID	Document Number	Publication Date	Patentee	Class	Subclass	Filing Date If Appropriate
/A.B./	1.	2014/0072978	3/13/2014	Maples et al.			
/A.B./	2.	2009/0017453	1/15/2009	Maples et al.			
/A.B./	3.	2009/0011472	01/08/2009	Nelson et al.			

Foreign Patent Documents or Published Foreign Patent Applications

Examiner Initial	Desig. ID	Document Number	Publication Date	Country or Patent Office	Class	Subclass	Translation Yes	Translation No
/A.B./	4.	WO08/002920	1/3/2008	WIPO				

Other Documents (include Author, Title, Date, and Place of Publication)

Examiner Initial	Desig. ID	Document
/A.B./	5.	Office Action in corresponding Application Number 12/173,020, dated October 3, 2016, pages 1-31
/A.B./	6.	Reply to Action of October 3, 2016, in corresponding Application Number 12/173,020, filed October 31, 2016, pages 1-21
/A.B./	7.	Office Action in corresponding Application Number 11/778,018, dated October 11, 2016, pages 1-29
/A.B./	8.	McDowell DG, Burns NA, Parkes HC. Localised sequence regions possessing high melting temperatures prevent the amplification of a DNA mimic in competitive PCR. Nucleic Acids Res. 1998 Jul 15; 26(14):3340-7
/A.B./	9.	Australian Office Action in corresponding Application Number 2015202439, dated September 28, 2016, pages 1-4
/A.B./	10.	Buck et al., “Design Strategies and Performance of Custom DNA Sequencing Primers,” BioTechniques, 27:528-536 (1999)
/A.B./	11.	Hite et al., “Factors affecting fidelity of DNA synthesis during PCR amplification of d(C-A),n-d(G-T)n microsatellite repeats,” Nucleic Acids Research, 1996, 24: 2429-34 (1996)

Examiner Signature /Angela Bertagna/	Date Considered 11/22/2016
EXAMINER: Initials citation considered. Draw line through citation if not in conformance and not considered. Include copy of this form with next communication to applicant.	

Substitute Disclosure Form

EAST Search History

EAST Search History (Prior Art)

Ref #	Hits	Search Query	DBs	Default Operator	Plurals	Time Stamp
S142	2	"5744311".pn. "5712124".pn.	USPAT	OR	OFF	2016/11/22 10:48
S143	4	us-20030138800-$.did. us-20090011472-$.did. us-20140072978-$.did. us-20090017453-$.did.	US-PGPUB	OR	OFF	2016/11/22 10:49
S144	10	(ionian).as. and (nicking or nick or nicked or nickase)	US-PGPUB; USPAT; FPRS; EPO; JPO; DERWENT	OR	OFF	2016/11/23 09:52
S149	10	((rich or richard) near2 roth).in. and nick$.clm.	US-PGPUB; USPAT; FPRS; EPO; JPO; DERWENT	OR	OFF	2016/11/23 09:53
S148	5	(jarrod near2 provins).in. and nick$.clm.	US-PGPUB; USPAT; FPRS; EPO; JPO; DERWENT	OR	OFF	2016/11/23 09:53
S147	16	(andrew near2 miller).in. and nick$.clm.	US-PGPUB; USPAT; FPRS; EPO; JPO; DERWENT	OR	OFF	2016/11/23 09:53
S146	4	(rebecca near2 holmberg).in. and nick$.clm.	US-PGPUB; USPAT; FPRS; EPO; JPO; DERWENT	OR	OFF	2016/11/23 09:53
S145	4	(brian near2 maples).in. and nick$.clm.	US-PGPUB; USPAT; FPRS; EPO; JPO; DERWENT	OR	OFF	2016/11/23 09:53
S150	5	(jeffrey near2 mandell).in. and nick$.clm.	US-PGPUB; USPAT; FPRS; EPO; JPO; DERWENT	OR	OFF	2016/11/23 09:54
S155	5	S149 not S148 not S147 not S145 not S144	US-PGPUB; USPAT; FPRS; EPO; JPO; DERWENT	OR	OFF	2016/11/23 09:55
S154	1	S148 not S147 not S145 not S144	US-PGPUB; USPAT; FPRS; EPO; JPO;	OR	OFF	2016/11/23 09:55

			DERWENT			
S153	12	S147 not S145 not S144	US-PGPUB; USPAT; FPRS; EPO; JPO; DERWENT	OR	OFF	2016/11/23 09:55
S152	0	S146 not S145 not S144	US-PGPUB; USPAT; FPRS; EPO; JPO; DERWENT	OR	OFF	2016/11/23 09:55
S151	1	S145 not S144	US-PGPUB; USPAT; FPRS; EPO; JPO; DERWENT	OR	OFF	2016/11/23 09:55
S156	1	S150 not S149 not S148 not S147 not S145 not S144	US-PGPUB; USPAT; FPRS; EPO; JPO; DERWENT	OR	OFF	2016/11/23 09:56
S158	1004	(primer near8 nicking)	US-PGPUB; USPAT; FPRS; EPO; JPO; DERWENT	OR	ON	2016/11/23 10:07
S157	828	(primer near8 nicking)	US-PGPUB; USPAT; FPRS; EPO; JPO; DERWENT	OR	OFF	2016/11/23 10:07
S159	1122	(primer near8 nick or nicked or nicking) same (isothermal$ or (constant adj1 temperature))	US-PGPUB; USPAT; FPRS; EPO; JPO; DERWENT	OR	ON	2016/11/23 10:16
S160	1105	(primer near3 nick or nicked or nicking) same (isothermal$ or (constant adj1 temperature))	US-PGPUB; USPAT; FPRS; EPO; JPO; DERWENT	OR	ON	2016/11/23 10:17
S161	231	(primer near3 nick or nicked or nicking near5 ((non adj1 complementary) or "not complementary" or tail or "5'" or tag)) same (isothermal$ or (constant adj1 temperature))	US-PGPUB; USPAT; FPRS; EPO; JPO; DERWENT	OR	ON	2016/11/23 10:39
S162	23	(primer near3 (nick or nicked or nicking) near5 ((non adj1 complementary) or "not complementary" or tail or "5'" or tag)) same (isothermal$ or (constant adj1 temperature))	US-PGPUB; USPAT; FPRS; EPO; JPO; DERWENT	OR	ON	2016/11/23 10:40
S165	85	S164 not S163	US-PGPUB; USPAT; FPRS; EPO; JPO; DERWENT	OR	ON	2016/11/23 10:50
S164	108	(primer near3 (nick or nicked or nicking) near5 ((non adj1 complementary) or "not complementary" or tail or "5'" or tag or tailed or tagged))	US-PGPUB; USPAT; FPRS; EPO; JPO;	OR	ON	2016/11/23 10:50

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S163	23	(primer near3 (nick or nicked or nicking) near5 ((non adj1 complementary) or "not complementary" or tail or "5'" or tag or tailed or tagged)) same (isothermal$ or (constant adj1 temperature))	US-PGPUB; USPAT; FPRS; EPO; JPO; DERWENT	OR	ON	2016/11/23 10:50
L1	10	(ionian).as. and (nicking or nick or nicked or nickase)	US-PGPUB; USPAT; FPRS; EPO; JPO; DERWENT	OR	OFF	2016/12/07 06:26
L4	16	(andrew near2 miller).in. and nick$.clm.	US-PGPUB; USPAT; FPRS; EPO; JPO; DERWENT	OR	OFF	2016/12/07 06:27
L3	4	(rebecca near2 holmberg).in. and nick$.clm.	US-PGPUB; USPAT; FPRS; EPO; JPO; DERWENT	OR	OFF	2016/12/07 06:27
L2	4	(brian near2 maples).in. and nick$.clm.	US-PGPUB; USPAT; FPRS; EPO; JPO; DERWENT	OR	OFF	2016/12/07 06:27
L7	5	(jeffrey near2 mandell).in. and nick$.clm.	US-PGPUB; USPAT; FPRS; EPO; JPO; DERWENT	OR	OFF	2016/12/07 06:28
L6	10	((rich or richard) near2 roth).in. and nick$.clm.	US-PGPUB; USPAT; FPRS; EPO; JPO; DERWENT	OR	OFF	2016/12/07 06:28
L5	5	(jarrod near2 provins).in. and nick$.clm.	US-PGPUB; USPAT; FPRS; EPO; JPO; DERWENT	OR	OFF	2016/12/07 06:28
L11	109	(primer near3 (nick or nicked or nicking) near5 ((non adj1 complementary) or "not complementary" or tail or "5'" or tag or tailed or tagged))	US-PGPUB; USPAT; FPRS; EPO; JPO; DERWENT	OR	ON	2016/12/07 06:30
L10	24	(primer near3 (nick or nicked or nicking) near5 ((non adj1 complementary) or "not complementary" or tail or "5'" or tag or tailed or tagged)) same (isothermal$ or (constant adj1 temperature))	US-PGPUB; USPAT; FPRS; EPO; JPO; DERWENT	OR	ON	2016/12/07 06:30
L9	24	(primer near3 (nick or nicked or nicking) near5 ((non adj1 complementary) or "not complementary" or tail or "5'" or tag)) same (isothermal$ or (constant adj1 temperature))	US-PGPUB; USPAT; FPRS; EPO; JPO; DERWENT	OR	ON	2016/12/07 06:30
L8	232	(primer near3 nick or nicked or nicking near5 ((non adj1 complementary) or	US-PGPUB; USPAT;	OR	ON	2016/12/07 06:30

		"not complementary" or tail or "5'" or tag)) same (isothermal$ or (constant adj1 temperature))	FPRS; EPO; JPO; DERWENT			

EAST Search History (Interference)

Ref #	Hits	Search Query	DBs	Default Operator	Plurals	Time Stamp
L15	16	(andrew near2 miller).in. and nick$.clm.	US-PGPUB; USPAT	OR	OFF	2016/12/07 06:38
L14	4	(rebecca near2 holmberg).in. and nick$.clm.	US-PGPUB; USPAT	OR	OFF	2016/12/07 06:38
L13	4	(brian near2 maples).in. and nick$.clm.	US-PGPUB; USPAT	OR	OFF	2016/12/07 06:38
L12	3	(ionian).as. and (nicking or nick or nicked or nickase)	US-PGPUB; USPAT	OR	OFF	2016/12/07 06:38
L21	23	(primer near3 (nick or nicked or nicking) near5 ((non adj1 complementary) or "not complementary" or tail or "5'" or tag or tailed or tagged)) same (isothermal$ or (constant adj1 temperature))	US-PGPUB; USPAT	OR	ON	2016/12/07 06:39
L20	23	(primer near3 (nick or nicked or nicking) near5 ((non adj1 complementary) or "not complementary" or tail or "5'" or tag)) same (isothermal$ or (constant adj1 temperature))	US-PGPUB; USPAT	OR	ON	2016/12/07 06:39
L19	201	(primer near3 nick or nicked or nicking near5 ((non adj1 complementary) or "not complementary" or tail or "5'" or tag)) same (isothermal$ or (constant adj1 temperature))	US-PGPUB; USPAT	OR	ON	2016/12/07 06:39
L18	5	(jeffrey near2 mandell).in. and nick$.clm.	US-PGPUB; USPAT	OR	OFF	2016/12/07 06:39
L17	10	((rich or richard) near2 roth).in. and nick$.clm.	US-PGPUB; USPAT	OR	OFF	2016/12/07 06:39
L16	5	(jarrod near2 provins).in. and nick$.clm.	US-PGPUB; USPAT	OR	OFF	2016/12/07 06:39
L22	100	(primer near3 (nick or nicked or nicking) near5 ((non adj1 complementary) or "not complementary" or tail or "5'" or tag or tailed or tagged))	US-PGPUB; USPAT	OR	ON	2016/12/07 06:40

12/7/2016 6:42:13 AM
C:\Users\abertagna\Documents\EAST\Workspaces\14067620\14067620.wsp

Issue Classification	Application/Control No.	Applicant(s)/Patent Under Reexamination
	14067620	MAPLES ET AL.
	Examiner	Art Unit
	ANGELA M BERTAGNA	1637

CPC

Symbol					Type	Version
C12Q		1	/	686	F	2013-01-01
C12Q		1	/	6844	I	2013-01-01
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CPC Combination Sets

Symbol					Type	Set	Ranking	Version
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NONE (Assistant Examiner)	(Date)	Total Claims Allowed: 35	
/ANGELA M BERTAGNA/ Primary Examiner.Art Unit 1637 (Primary Examiner)	12/7/16 (Date)	O.G. Print Claim(s) 1	O.G. Print Figure NONE

U.S. Patent and Trademark Office

Part of Paper No. 20161122

Issue Classification	Application/Control No.	Applicant(s)/Patent Under Reexamination
	14067620	MAPLES ET AL.
	Examiner	**Art Unit**
	ANGELA M BERTAGNA	1637

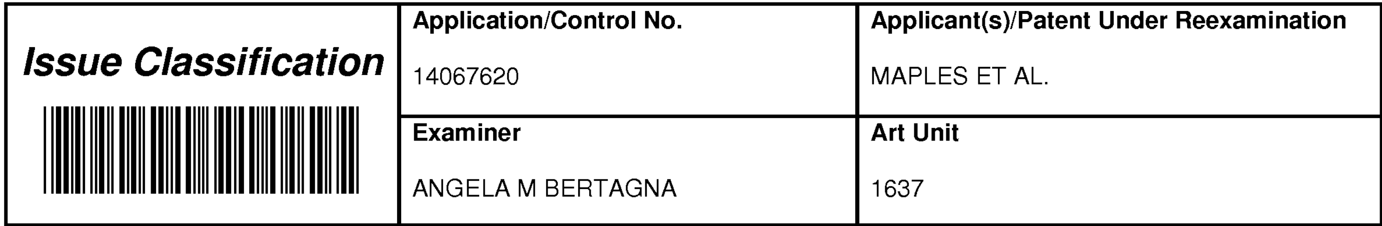
US ORIGINAL CLASSIFICATION		INTERNATIONAL CLASSIFICATION									
CLASS	SUBCLASS	CLAIMED					NON-CLAIMED				
		C	1	2	Q	1 / 68 (2006.01.01)					

CROSS REFERENCE(S)

CLASS	SUBCLASS (ONE SUBCLASS PER BLOCK)				

NONE (Assistant Examiner) (Date)		**Total Claims Allowed:** 35
/ANGELA M BERTAGNA/ Primary Examiner.Art Unit 1637 12/7/16 (Primary Examiner) (Date)	O.G. Print Claim(s) 1	O.G. Print Figure NONE

U.S. Patent and Trademark Office Part of Paper No. 20161122



Issue Classification	Application/Control No.	Applicant(s)/Patent Under Reexamination
	14067620	MAPLES ET AL.
	Examiner	**Art Unit**
	ANGELA M BERTAGNA	1637

☐ **Claims renumbered in the same order as presented by applicant** ☐ **CPA** ☒ **T.D.** ☐ **R.1.47**

Final	Original	Final	Original	Final	Original	Final	Original	Final	Original	Final	Original	Final	Original	Final	Original
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	2		18		34		50		66	16	82	26	98		
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	8		24		40		56	6	72	20	88	32	104		
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	15		31		47		63	13	79	23	95				
	16		32		48		64	14	80	24	96				

NONE (Assistant Examiner)	(Date)	**Total Claims Allowed:** 35	
/ANGELA M BERTAGNA/ Primary Examiner.Art Unit 1637 (Primary Examiner)	12/7/16 (Date)	O.G. Print Claim(s) 1	O.G. Print Figure NONE

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PTO/SB/30 (07-09)
Approved for use through 07/31/2012. OMB 0651-0031
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Request for Continued Examination (RCE) Transmittal

Address to:
Mail Stop RCE
Commissioner for Patents
P.O. Box 1450
Alexandria, VA 22313-1450

Application Number	14/067,620
Filing Date	October 30, 2013
First Named Inventor	Brian K. Maples
Art Unit	1637
Examiner Name	Angela Marie Bertagna
Attorney Docket Number	30171-0025002

This is a Request for Continued Examination (RCE) under 37 CFR 1 .114 of the above-identified application.
Request for Continued Examination (RCE) practice under 37 CFR 1.114 does not apply to any utility or plant application filed prior to June 8, 1995, or to any design application. See Instruction Sheet for RCEs (not to be submitted to the USPTO) on page 2.

1. Submission required under 37 CFR 1.114 Note: If the RCE is proper, any previously filed unentered amendments and amendments enclosed with the RCE will be entered in the order in which they were filed unless applicant instructs otherwise. If applicant does not wish to have any previously filed unentered amendment(s) entered, applicant must request non-entry of such amendment(s).
   - a. ☐ Previously submitted. If a final Office action is outstanding, any amendments filed after the final Office action may be considered as a submission even if this box is not checked.
     - i. ☐ Consider the arguments in the Appeal Brief or Reply Brief previously filed on ____
     - ii. ☐ Other ____
   - b. ☒ Enclosed
     - i. ☐ Amendment/Reply
     - ii. ☐ Affidavit(s)/ Declaration(s)
     - iii. ☒ Information Disclosure Statement (IDS)
     - iv. ☐ Other ____
2. Miscellaneous
   Suspension of action on the above-identified application is requested under 37 CFR 1.103(c) for a
   - a. ☐ period of ____ months. (Period of suspension shall not exceed 3 months; Fee under 37 CFR 1.17(i) required)
   - b. ☐ Other ____
3. Fees The RCE fee under 37 CFR 1.17(e) is required by 37 CFR 1.114 when the RCE is filed.
   The Director is hereby authorized to charge the following fees any underpayment of fees or credit any overpayments to
   - a. ☒ Deposit Account No. 06-1050 .
     - i. ☒ RCE fee required under 37 CFR 1.17(e)
     - ii ☐ Extension of time fee (37 CFR 1.136 and 1.17)
     - iii ☒ Other any deficiencies
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SIGNATURE OF APPLICANT, ATTORNEY, OR AGENT REQUIRED

Signature	/Ian J.S. Lodovice, Reg. No. 59,749/	Date	November 1, 2016
Name (Print/Type)	Ian J. Lodovice	Registration No.	59,749

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This collection of information is required by 37 CFR 1.114. The information is required to obtain or retain a benefit by the public which is to file (and by the USPTO to process) an application. Confidentiality is governed by 35 U.S.C. 122 and 37 CFR 1.11 and 1.14. This collection is estimated to take 12 minutes to complete, including gathering, preparing, and submitting the completed application form to the USPTO. Time will vary depending upon the individual case. Any comments on the amount of time you require to complete this form and/or suggestions for reducing this burden, should be sent to the Chief Information Officer, U.S. Patent and Trademark Office, U.S. Department of Commerce, P.O. Box 1450, Alexandria, VA 22313-1450. DO NOT SEND FEES OR COMPLETED FORMS TO THIS ADDRESS. **SEND TO: Mail Stop RCE, Commissioner for Patents, P.O. Box 1450, Alexandria, VA 22313-1450.**
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IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

First Named Inventor	:	Brian K. Maples	Art Unit	:	1637
Patent No.	:	9,487,826	Examiner	:	Angela Marie Bertagna
Issue Date	:	November 8, 2016			
Serial No.	:	14/067,620	Conf. No.	:	4288
Filed	:	October 30, 2013			
Title	:	NICKING AND EXTENSION AMPLIFICATION REACTION FOR THE EXPONENTIAL AMPLIFICATION OF NUCLEIC ACIDS			

MAIL STOP RCE
Commissioner for Patents
P.O. Box 1450
Alexandria, VA 22313-1450

INFORMATION DISCLOSURE STATEMENT

Please consider the references listed on the enclosed PTO-SB-08 or Disclosure Form. Foreign patent documents and non-patent literature are enclosed; cited U.S. patents and patent application publications will be provided on request. A copy of a communication from a foreign patent office in a counterpart application is also enclosed.

This filing is being made with the filing of a Request for Continued Examination. No fee is required.

Apply any necessary charges or credits to deposit account 06-1050, referencing the above attorney docket number.

Please contact the undersigned if there are any questions regarding this statement.

Respectfully submitted,

Date: November 1, 2016

/Ian J.S. Lodovice, Reg. No. 59,749/
Ian J. Lodovice
Reg. No. 59,749

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Substitute Disclosure Form U.S. Department of Commerce Patent and Trademark Office	Attorney Docket No. 30171-0025002	Application No. 14/067,620
Information Disclosure Statement by Applicant (Use several sheets if necessary)	Applicant Ionian Technologies Inc.	
(37 CFR §1.98(b))	Filing Date October 30, 2013	Group Art Unit 1637

U.S. Patent Documents							
Examiner Initial	Desig. ID	Document Number	Publication Date	Patentee	Class	Subclass	Filing Date If Appropriate
	1.	2014/0072978	3/13/2014	Maples et al.			
	2.	2009/0017453	1/15/2009	Maples et al.			
	3.	2009/0011472	01/08/2009	Nelson et al.			

Foreign Patent Documents or Published Foreign Patent Applications								
Examiner Initial	Desig. ID	Document Number	Publication Date	Country or Patent Office	Class	Subclass	Translation	
							Yes	No
	4.	WO08/002920	1/3/2008	WIPO				

Other Documents (include Author, Title, Date, and Place of Publication)		
Examiner Initial	Desig. ID	Document
	5.	Office Action in corresponding Application Number 12/173,020, dated October 3, 2016, pages 1-31
	6.	Reply to Action of October 3, 2016, in corresponding Application Number 12/173,020, filed October 31, 2016, pages 1-21
	7.	Office Action in corresponding Application Number 11/778,018, dated October 11, 2016, pages 1-29
	8.	McDowell DG, Burns NA, Parkes HC. Localised sequence regions possessing high melting temperatures prevent the amplification of a DNA mimic in competitive PCR. Nucleic Acids Res. 1998 Jul 15; 26(14):3340-7
	9.	Australian Office Action in corresponding Application Number 2015202439, dated September 28, 2016, pages 1-4
	10.	Buck et al., "Design Strategies and Performance of Custom DNA Sequencing Primers," BioTechniques, 27:528-536 (1999)
	11.	Hite et al., "Factors affecting fidelity of DNA synthesis during PCR amplification of d(C-A),n-d(G-T)n microsatellite repeats," Nucleic Acids Research, 1996, 24: 2429-34 (1996)

Examiner Signature	Date Considered
EXAMINER: Initials citation considered. Draw line through citation if not in conformance and not considered. Include copy of this form with next communication to applicant.	

Substitute Disclosure Form

(12) INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(19) World Intellectual Property Organization
International Bureau

(43) International Publication Date
3 January 2008 (03.01.2008)

PCT

(10) International Publication Number
WO 2008/002920 A2

(51) International Patent Classification:
C12Q 1/68 (2006.01)

(21) International Application Number:
PCT/US2007/072136

(22) International Filing Date: 26 June 2007 (26.06.2007)

(25) Filing Language: English

(26) Publication Language: English

(30) Priority Data:
60/805,847 26 June 2006 (26.06.2006) US

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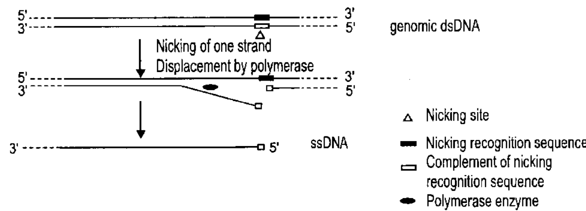
(74) Agents: **FANG, Carol A.** et al.; Townsend and Townsend and Crew, LLP., Two Embarcadero Center, Eighth Floor, San Francisco, California 94111-3834 (US).

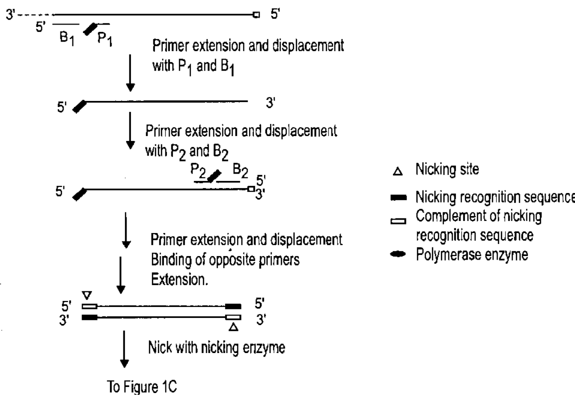
(81) Designated States *(unless otherwise indicated, for every kind of national protection available)*: AE, AG, AL, AM, AT, AU, AZ, BA, BB, BG, BH, BR, BW, BY, BZ, CA, CH, CN, CO, CR, CU, CZ, DE, DK, DM, DO, DZ, EC, EE, EG, ES, FI, GB, GD, GE, GH, GM, GT, HN, HR, HU, ID, IL, IN, IS, JP, KE, KG, KM, KN, KP, KR, KZ, LA, LC, LK, LR, LS, LT, LU, LY, MA, MD, ME, MG, MK, MN, MW, MX, MY, MZ, NA, NG, NI, NO, NZ, OM, PG, PH, PL, PT, RO, RS, RU, SC, SD, SE, SG, SK, SL, SM, SV, SY, TJ, TM, TN, TR, TT, TZ, UA, UG, US, UZ, VC, VN, ZA, ZM, ZW.

(84) Designated States *(unless otherwise indicated, for every kind of regional protection available)*: ARIPO (BW, GH, GM, KE, LS, MW, MZ, NA, SD, SL, SZ, TZ, UG, ZM, ZW), Eurasian (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European (AT, BE, BG, CH, CY, CZ, DE, DK, EE, ES, FI, FR, GB, GR, HU, IE, IS, IT, LT, LU, LV, MC, MT, NL, PL,

[Continued on next page]

(54) Title: METHODS FOR GENERATING TARGET NUCLEIC ACID SEQUENCES





(57) Abstract: The present invention provides methods of generating target nucleic acids for amplification using nicking enzymes and methods for amplifying the generated target nucleic acids.

WO 2008/002920 A2

PT, RO, SE, SI, SK, TR), OAPI (BF, BJ, CF, CG, CI, CM, GA, GN, GQ, GW, ML, MR, NE, SN, TD, TG).

Published:

— *without international search report and to be republished upon receipt of that report*

For two-letter codes and other abbreviations, refer to the "Guidance Notes on Codes and Abbreviations" appearing at the beginning of each regular issue of the PCT Gazette.

Methods for Generating Target Nucleic Acid Sequences

CROSS-REFERENCES TO RELATED APPLICATIONS

[0001] This application claims the benefit of U.S. Provisional Application No. 60/805,847, filed June 26, 2006, the disclosure of which is hereby incorporated by reference in its entirety for all purposes.

STATEMENT AS TO RIGHTS TO INVENTIONS MADE UNDER FEDERALLY SPONSORED RESEARCH OR DEVELOPMENT

[0002] NOT APPLICABLE

REFERENCE TO A "SEQUENCE LISTING," A TABLE, OR A COMPUTER PROGRAM LISTING APPENDIX SUBMITTED ON A COMPACT DISK.

[0003] NOT APPLICABLE

BACKGROUND OF THE INVENTION

[0004] Exponential strand displacement amplification (SDA) was disclosed in U.S. Patent No. 5,455,166 requiring an initial denaturation of the target into single-stranded DNA (ssDNA), generation of hemiphosphorothioate sites which allow single strand nicking by restriction enzymes and extension by a polymerase lacking 5'-3' exonuclease activity. U.S. Patent No. 5,624,825 disclosed the simultaneous detection of more than one target and the requirement of at least one modified deoxynucleoside triphosphate (dNTP).

[0005] U.S. Patent No. 5,648,211 describes the use of thermostable enzymes in SDA requiring either the generation of a hemimodified restriction site during amplification or the use of a substituted deoxynucleoside triphosphate. α-Boronated deoxynucleoside triphosphates, when incorporated into a double-stranded DNA (dsDNA), generates a restriction endonuclease recognition/cleavage site allowing a single nick in one DNA strand (U.S. Patent No. 5,702,926). An isothermal *in situ* strand displacement amplification utilizing exposure to dry heat, restriction endonuclease pretreatment and mild depurination is disclosed by Nuovo (Diagnostic Molec. Pathol., 9: 195-202 (2000)).

[0006] An abasic site endonuclease amplification assay was disclosed in U.S. Patent Application No. 2004/0101893. The use of this assay as a post amplification detection system in combination with other amplification systems, were also disclosed. All these assays require a denaturation step of dsDNA.

5 [0007] A few nucleases cut just one strand of DNA thereby introducing a nick into DNA (Besnier and Kong, *EMBO Reports*, 21: 782-786 (2001)). Most such proteins are involved in DNA repair and other DNA-related metabolism and cannot easily be used to manipulate DNA. They usually recognize long sequences and associate with other proteins to form active complexes that are difficult to manufacture (Higashitani *et al., J. Mol. Biol.*, 237: 388-4000
10 (1994)). Single strand nicking endonucleases which nick only one strand of the DNA double strands and traditional restriction endonucleases are listed and updated in the REBASE Database (Roberts *et al., Nucl. Acids Res.*, 31: 418-420 (2003)). Engineering of a nicking endonuclease has been described (Xu *et al, PNAS USA* 98: 12990-12995 (2001)). Isothermal assays using nicking enzymes, but still requiring a thermal denaturation step are described by
15 Ehses *et al., J. Biochem. Biophys.* 63:170-186 (2005).

[0008] What is needed in the art is an isothermal assay which combines the advantages of target nucleic acid cycling, without the requirement for dsDNA denaturation and the use of modified dTNPs, retaining binding stability of the probe, an exquisitely specific cleavage site, the possibility for essentially instantaneous and highly sensitive reporter detection and
20 the ability to directly combine detection with amplification procedures. Accordingly, there remains a need for compositions and methods that enable efficient detection of target nucleic acids with exquisite specificity. The present invention fulfills this need and others.

BRIEF SUMMARY OF THE INVENTION

25 [0009] The present invention provides methods and compositions for improved hybridization and mismatch discrimination by isothermal amplification. In the practice of the invention, dsDNA is amplified isothermally by single strand displacement in the presence of a polymerase and a nicking enzyme that cuts on strand allowing the isothermal strand displacement. The method involves 1) the isolation of the target nucleic acids from a sample,
30 2) providing a mixture comprising a) a nicking endonuclease enzyme, b) a nucleic acid polymerase, c) deoxynucleosidetriphosphates and at least one primer which is complementary to a region at the 3'-end of a target fragment and further wherein each primer

has a recognition sequence for the nicking endonuclease upstream of the 5'-end, allowing a time sufficient to generate nicks in one strand, the initiation points for isothermal strand displacement and the generation of reaction products.

[0010] One embodiment of the invention provides a method for generating a target nucleic acid sequence for amplification. The method comprises (a) providing a double stranded target sequence; (b) nicking one strand of said target sequence with a nicking enzyme, thereby generating the target nucleic acid sequence without thermal denaturation of the double stranded target sequence. The recognition site of the nicking enzyme: (i) is at least 6 nucleotides in length, (ii) is present in one strand of the target sequence about 1 to about 50 times, or (iii) comprises a combination of (i) and (ii). In some embodiments, the recognition site of the nicking enzyme is at least 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, or 16 nucleotides in length. In some embodiments, the recognition site of the nicking enzyme is present in one strand of the target sequence about 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17,18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, 30, 31, 32, 33, 34, 35, 36, 37, 38, 39, 40, 41, 42, 43, 44, 45, 46, 47, 48, 49, or 50 times. In some embodiments, the nicking enzyme is a type IIS nicking enzyme (*e.g.*, a nicking enzyme selected from: Nt.BbvCI, Nb.BsmI, N. BbvC IA, N.BbvC IB, N.BstNB I, N.Alw I, Nb.Bpu101, N.Bst9I, NMlyI, R.BbvCI, Nb.SapI-1 (variant 33) and Nb.SapI-1 (E250K)). In some embodiments, the nicking enzyme is a modified type IIS nicking enzyme.

[0011] Another embodiment of the invention provides a method for amplifying a target nucleic acid sequence. The method comprises (a) generating a target nucleic acid sequence according to the method of claim 1; (b) contacting a first extension primer and a first bumper primer with the target nucleic acid sequence under conditions sufficient to allow first extension primer to hybridize to the target nucleic acid sequence and for the first bumper primer to hybridize to the target nucleic sequence at a site 5' to the binding site of the first extension primer, wherein the 3' end of the first extension primer comprises a target binding sequence and the 5' end of the first extension primer comprises: (i) a recognition sequence for the nicking enzyme and (ii) a sequence which is complementary to the target nucleic acid, (c) simultaneously extending the first extension primer and the first bumper primer with a polymerase to produce a first extension product and a first bumper extension product that displaces the first extension product; (d) contacting a second extension primer and a second bumper primer with the displaced first extension product under conditions sufficient to allow the second extension primer to hybridize to the first extension product and for the second

bumper primer to hybridize to the first extension product at a site 5' to the binding site of the second extension primer, wherein the 3' end of the second extension primer comprises a sequence that binds to the first extension product and the 5' end of the second extension primer comprises: (i) a recognition sequence for the nicking enzyme and (ii) a sequence which is complementary to the target nucleic acid; and (e) simultaneously extending the second extension primer and the second bumper primer with the polymerase to produce a second extension product and a second bumper extension product that displaces the second extension product, thereby generating an amplified target sequence. In some embodiments, the polymerase is a DNA polymerase without 5'→3' exonuclease activity (*e.g.*, a polymerase selected from: Bst DNA Polymerase Large Fragment, Bca DNA polymerase, Klenow fragment of DNA polymerase I, Phi29 DNA polymerases, Sequenase 2.0 T7 DNA Polymerase and T5 DNA polymerase). In some embodiments, the method further comprises (f) contacting the first extension primer to the second extension product under conditions sufficient to allow the first extension primer to hybridize to the second extension product and extending the first extension primer with the polymerase to generate a double stranded product comprising restriction sites recognized by the nicking enzyme; (g) contacting the double stranded product with the nicking enzyme under conditions sufficient to allow the nicking enzyme to cleave a single strand of the double stranded product, thereby generating a nicked double stranded product with a nick site on each strand; (h) contacting the first and second extension primer with the nicked double stranded product under conditions sufficient to allow the first and second extension primers to hybridize to the nicked double stranded product; and (i) extending the first and second extension primers with a polymerase, thereby releasing single stranded amplified target sequences into solution. In some embodiments, the method further comprises(h) detecting the amplified target sequence. In some embodiments, the amplified target sequence is detected by: (i) contacting the amplified target sequence with AP site probe and an AP endonuclease under conditions sufficient to allow the AP site probe to hybridize to the amplified target nucleic acid, wherein the AP site probe comprises an oligonucleotide NA that hybridizes to the amplified target nucleic acid and a functional tail R comprising a detectable reporter group, the functional tail R attached via a phosphodiester bond of a phosphate group to the 3′ terminal nucleotide of the NA, wherein the reporter group is not detected when the functional tail R is attached to the NA; and (j) incubating the amplified target nucleic acid sequence, the AP site probe, and the AP endonuclease under conditions sufficient to allow the AP endonuclease to cleave the phosphodiester bond attaching the functional tail R to the 3′ terminal nucleotide of the NA, wherein the AP

endonuclease preferentially cleaves the phosphodiester bond attaching the tail R to the NA when the NA is hybridized with a complementary target nucleic acid sequence in comparison to when the NA is unhybridized or hybridized to a non-complementary nucleic acid; and (k) detecting the reporter group on the cleaved functional tail R, whereby the amplified target
5 nucleic acid sequence is detected. In some embodiments, the method further comprises contacting the amplified target nucleic acid sequence with an enhancer oligonucleotide, wherein the 5′-end of the enhancer oligonucleotide hybridizes to the amplified target nucleic acid sequence about 0 to about 5 bases 3′ to the site where the AP site probe hybridizes to the amplified target nucleic acid sequence. In some embodiments, the 5’-end of the AP site
10 probe is covalently linked to the 3’-end of the enhancer. In some embodiments, a quencher molecule is attached to the 5′ end of the NA of the AP site probe via a non-cleavable linker. In some embodiments, cleavage of the phosphodiester bond results in a hybridized NA having a free 3′-OH. In some embodiments, the amplified target nucleic acid sequence, the AP site probe, and the AP endonuclease are incubated under conditions sufficient to
15 simultaneously allow the AP endonuclease to cleave the phosphodiester bond of the AP site probe and the polymerase to extend the cleaved AP site probe in a template-specific manner. In some embodiments, the NA of the AP site probe is 3-200, 5-150, 7-100, 10-50, 15-40, or 20-30 nucleotides in length. In some embodiments, the AP site probe further comprises at least one modified base. In some embodiments, the functional tail R is attached to the
20 phosphate group through a hydroxyprolinol linker. In some embodiments, the reporter group is a fluorophore. In some embodiments, the AP endonuclease is a Class II AP endonuclease (*e.g.*, an *E.coli* Endonuclease IV).

[0012] A further embodiment of the invention provides kits comprising at least one of the nicking enzymes, primers, probes, and DNA polymerases described herein.

25 BRIEF DESCRIPTION OF THE DRAWINGS

[0013] Figure 1 is a diagram illustrating the method of the invention to perform true isothermal amplification of dsDNA. Figure 1A illustrates the isothermal generation of ssDNA template. Figure 1B illustrates the initial steps of SDA and Figure 1C illustrates the exponential cycles in SDA.

30 **[0014]** Figure 2 shows an example of an endonuclease IV signal detection system.

[0015] Figure 3 shows real-time homogeneous amplification (SDA) and detection of *Mycobacterium tuberculosis* DNA using an endonuclease IV probe. Fluorescence is plotted versus time for various amounts of input target.

[0016] Figure 4 shows post-amplification genotype analysis of isothermal 1-step SDA product on NanoChip® microarray.

[0017] Figure 5 shows Real time genotype analysis of isothermal 1-step SDA products from human genomic DNA using MGB Eclipse® genotyping probes (Nanogen, Bothell, WA) Fig. 5A represents the analysis of homozygous wild type samples. Fig. 5B represents analysis of mutant samples, respectively, Fig. 5C represents the analysis of a heterozygous sample. Fig 5D represents the no template control.

DETAILED DESCRIPTION OF THE INVENTION

I. General

[0018] The present invention provides assay methods that combine the advantages of nucleic acid amplification cycling with a nicking enzyme that cuts one strand of dsDNA allowing the isothermal strand displacement generation of single strand DNA eliminating the need for thermal denaturation, and providing methods with efficient, flexible and simpler amplification protocols.

II. Definitions

[0019] As use in herein a "nick" is a point in a double stranded DNA molecule where there is no phosphodiester bond between adjacent nucleotides of one strand. A nick is typically induced by damage or caused by enzyme action.

[0020] A "nicking endonuclease" or "nicking enzyme" is an enzyme that specifically introduces a nick in one of the strands in a double-stranded nucleic acid (*e.g.*, DNA).

[0021] A "restriction endonuclease" is a class of bacterial enzymes that cut both strands of DNA at specific sites.

[0022] An "amplicon sequence" is the target sequence that is exponentially amplified and contains primer specific sequences.

[0023] "Isothermal SDA amplification" or "isothermal strand displacement amplification" as used in here refers to amplification where no thermal denaturation of double stranded

DNA (dsDNA) (*e.g.*, at 95°C or other elevated temperature) is used to generate single stranded DNA (ssDNA). The isothermal SDA amplification of the present refers the generation of ssDNA by a polymerase with strand displacement ability starting from a nicking site, allowing amplification by the polymerase.

5 **[0024]** An "extension primer" is a primer that is extended during an SDA reaction.

[0025] A "bumper primer" is a primer used to displace primer extension products in SDA reaction. The bumper primer anneals to a target sequence upstream of the extension primer such that extension of the bumper primer displaces the downstream extension primer and its extension product.

10 **[0026]** An abasic site is an naturally occurring Apurinic/Apyrimidinic (AP) site in a nucleic acid sequence or a synthetic linker that is recognized and cleaved by Class II AP endonucleases when it appears in double stranded DNAs.

[0027] As used herein, an AP endonuclease refers to an enzyme that binds to and cleaves the phosphodiester backbone at an abasic (AP) site on a nucleic acid strand in a double 15 stranded DNA. Preferred AP endonucleases cleave the phosphodiester backbone on the 5′ side of the AP site via a hydrolytic mechanism that provides a free 3′-OH group that serves as a substrate for DNA polymerases.

III. Isothermal Strand Displacement Amplification

[0028] SDA amplification after generation of a ssDNA by thermal denaturation has been 20 disclosed (U.S. Patent Nos.5,455,166; 5,624,825; 5,624,825 and Walker *et al., Nucl. Acids Res.*, 24: 348-353 (1996)) as illustrated in Figure 1b) and c). The generation of ssDNA after nicking by a nicking endonuclease and displacement by DNA polymerase lacking 5' to 3' exonuclease activity is illustrated in Figure 1a).

[0029] In contrast to traditional "isothermal SDA" which require at least one thermal 25 denaturation step at about 95°C to generate single stranded DNA (ssDNA), the isothermal SDA of the invention generates ssDNA isothermally by polymerase strand displacement and, accordingly eliminates the thermal denaturation step. The isothermal generation of single stranded DNA, requires the involvement of a nicking enzyme that catalyzes a nick in one of the DNA strands, the initiation point for strand displacement by the polymerase.

[0030] According to the methods of the invention, a single stranded target nucleic acid sequence is generated by contacting a double-stranded target nucleic acid sequence (*e.g.*, dsDNA) with a nicking enzyme (*e.g.*, a type IIs modified nicking enzyme as described herein).

5 **[0031]** Three issues are of importance to facilitate the production of ssDNA for SDA: i) the frequency of the nicking enzyme recognition sequence in the target gene sequence, ii) the length of the nicking recognition sequence and iii) the orientation of the nicking enzyme's recognition sequence. The frequency of the nicking enzyme recognition sequences in the target is such that the strand displaced generated ssDNA sequences are equal or greater than 10 that of the amplicon sequence. The nicking enzyme recognition site is at least 6, 7, 8, or 9 nucleotides in length. In some embodiments, the nicking enzyme recognition site occurs in the target sequence about 1 to about 50, about 2 to about 40, about 3 to about 35, about 4 to about 30, about 5 to about 25, about 6 to about 20, about 10 to about 15 times. In some embodiments, the nicking enzyme recognition site is located about 1 to about 10,000, about 2 15 to about 5,000, about 3 to about 2,500, about 4 to about 1250, about 5 to about 1,000, about 10 to about 500, about 15 to about 250, about 20 to about 150 nucleotides from the binding site for a probe to detect the amplified target nucleic acid. In some embodiments, the nicking enzyme is selected from the enzymes disclosed in Table 1.

[0032] In some embodiments, the recognition site sequence of the nicking enzyme is 20 located upstream of the 5'-end of the targeted sequence. Strand displacement from the nicking site, by the polymerase lacking 5' to 3' exonuclease activity with good strand displacement characteristics, will therefore produce single strand targets that will include the probe recognition sequence.

[0033] In one embodiment the isothermal SDA of the present invention requires at least 25 one nicking endonuclease. A preferred embodiment utilizes a first and a second nicking enzymes, one set of primers containing a recognition sequence located upstream of the 5'-end of the probe sequence for one of said nicking enzymes.

[0034] In one embodiment target nucleic acid by strand displacement amplification is performed by using a thermophilic nicking enzyme, a thermophilic DNA polymerase and a 30 thermophilic endonuclease IV. In another embodiment at least one of the three enzymes is thermophilic. The use of a thermophilic DNA polymerase and a thermophilic restriction endonuclease was previously disclosed in U.S. Patent No. 5,648,211.

[0035] In another embodiment SDA is performed in the presence of a nicking enzyme, a restriction enzyme, generated hemimodified restriction site and a polymerase lacking 5'-3' exonuclease activity. A hemimodified restriction site is generated during amplification utilizing at least one dNTP selected from dNTPαS (U.S. Patent No. 5,455,166), a modified
5 dNTP (U.S. Patent No. 5,624,825) or α-boronated deosynucleoside triphosphate (U.S. Patent No. 5,648,211). In the above embodiment, the nicking enzyme generates nicks for the initiation of strand displacement, allowing isothermal generation of single stranded DNA target. Amplification primers containing a restriction recognition sequence upstream of the 5'-end of the primer recognition sequence, allow the generation of hemimodified restriction
10 modified site using an appropriate modified dTNP.

A. Nicking Enzymes

[0036] Nicking enzymes can be isolated or genetically engineered from restriction enzymes (Xu et al, PNAS 98: 12990-12995 (2001)). A number of nicking enzymes are available either commercially or have been disclosed. The following Table contains a list of nicking enzymes
15 useful to perform the isothermal amplification of the current inventions.

Table I. Nicking enzymes and their specificity.

Enzyme	Nicking Specificity	Source
Nb.BsmI	5'...GAATGCN...3' 3'...CTTAC▲GN...5'	http://www.neb.com
N. BbvC IA	5'...GC▼TGAGG...3' 3'...CGACTCC...5'	http://www.neb.com
N.BbvC IB	5'...CC▼TCAGC...3' 3'...GGAGTCG...5'	http://www.neb.com
N.BstNB I	5'...GAGTCNNNN▼N...3' 3'...CTCAGNNNNN...5'	http://www.neb.com
N.Alw I	5'...GGATCNNNN▼N...3' 3'...CCTAGNNNNN...5'	http://www.neb.com
Nb.Bpu101	5'...GC▼TNAGG...3' 3'...CGANTCC...5'	http://www.fermentas.com U.S. Patent No. 6,867,028
N.Bst9I	5'...GAGTCNNNN▼N...3' 3'...CACAGNNNNN...5'	http://www.sibenzyme.com
NMlyI	5'...GAGTCNNNNN▼N...3 3'...CTCAGNNNNNN...5'	Besnier & Kong, EMBO Reports, 21: 782-786 (2001).
R.BbvCI	5'...CCTCAGC...3' 3'...GG▲AGTCG...5' 5'...CC▼TCAGC...3' 3'...GGAGTCG...5'	Heiter et al., JMB., 348:631-640 (2005)
Nb.SapI-1	5'...GCTCTTCNNNNN...3'	Samuelson et al., NAR., 32:3661-

(variant 33)	3'...CGAGAAGNNNN▲N...5'	3671 (2004)
Nb.SapI-1 (E250K)	5'...GCTCTTCN▼N ...3' 3'...CGAGAAGNN...5'	

▲ or ▼ indicates the nicking position in the sequence.

[0037] There are more than eighty type of IIA/IS restriction endonuclease with different known recognition specificities. A novel genetic screening method was devised to convert type IIS restriction endonucleases into strand-specific nicking endonucleases (Zhu et al, J Mol Biol., 337: 573-83 (2005)). A selected preferred list of these restriction enzymes and their recognition sequences are listed in Table II. These type IIS restriction enzymes can potentially be converted by the Zhu et al protocol to nicking enzymes.

Table 2. Palindromic Restriction Endonucleases.

Enzymes	**Recognition Sequence**	**Enzymes**	**Recognition Sequence**
Aar I	5'...CACCTGC$(N)_4$▼...3' 3'...GTGGACG$(N)_8$▲...5'	BseY I	5'...C▼CCAGC...3' 3'...GGGTC▲G...5'
Ace III	5'...CAGCTC$(N)_7$▼...3' 3'...GTCGAG$(N)_{11}$▲...5'	Bsg I	5'...GTGCAG$(N)_{16}$▼...3' 3'...CCAGAG$(N)_{14}$▲...5'
Acu I	5'...CTGAAG$(N)_{16}$▼...3' 3'...GACTTC$(N)_{14}$▲...5'	Bsm I	5'...GAATGCN▼...3' 3'...CCAGA▲GN...5'
Alw I	5'...GGATC$(N)_4$▼...3' 3'...CCTAG$(N)_5$▲...5'	BspM I	5'...ACCTGC$(N)_4$▼...3' 3'...TGGACG$(N)_8$▲...5'
Bbr7 I	5'...GAAGAC$(N)_7$▼...3' 3'...CTTCTG$(N)_{11}$▲...5'	BsrB I	5'...CCG▼CTC...3' 3'...GGC▲GAG...5'
Bbs I	5'...GAAGAC$(N)_2$▼...3' 3'...CTTCTG$(N)_6$▲...5'	BsrD I	5'...GCAATG$(N)_2$▼...3' 3'...CGTTAC▲N...5'
Bbv I	5'...GCAGC$(N)_8$▼...3' 3'...CGTCG$(N)_{12}$▲...5'	BssS I	5'...C▼ACGAG$(N)_4$...3' 3'...GTGCT▲C$(N)_8$...5'
BbvC I	5'...CC▼TCAGC...3' 3'...GGAGT▲CG...5'	BtgZ I	5'...GCGATG$(N)_{10}$▼...3' 3'...CGCTAC$(N)_{14}$▲...5'
Bcc I	5'...CCATC$(N)_4$▼...3' 3'...GGTAG(N)5▲...5'	Bts I	5'...GCAGTG$(N)_2$▼...3' 3'...CGTCAC▲N...5'
BciV I	5'...GTATCC$(N)_6$▼...3' 3'...CATAGG$(N)_5$▲...5'	CstM I	5'...AAGGAG$(N)_{20}$▼...3' 3'...TTCCTC$(N)_{18}$▲...5'
BfuA I	5'...ACCTGC$(N)_4$▼...3' 3'...TGGACG$(N)_8$▲...5'	Drd II	5'...GAACCA▼...3' 3'...CTTGGT▲...5'
BmgB I	5'...CAC▼GTC...3' 3'...GTG▲CAG...5'	Ear I	5'...CTCTTC$(N)_1$▼...3' 3'...GAGAAG$(N)_4$▲...5'
Bmr I	5'...ACTGGG$(N)_5$▼...3' 3'...TGACCC$(N)_4$▲...5'	Eci I	5'...GGCGGA$(N)_{11}$▼...3' 3'...CCGCCT$(N)_9$▲...5'
Bpm I	5'...CTGGAG$(N)_{16}$▼...3' 3'...GACCTC$(N)_{14}$▲...5'	EcoP15 I	5'...CAGCAG$(N)_{25}$▼...3' 3'...GTCGTC$(N)_{27}$▲...5'
Bpu10 I	5'...CC▼TNAGC...3 3'...GGANT▲CG...5'	Pfl1108 I	5'...TCGTAG▼...3' 3'...AGCATC$_7$▲...5'
BpuE I	5'...CTTGAG$(N)_{16}$▼...3'	RleA I	5'...CCCACA$(N)_{12}$▼...3'

	3'...GAACAC$(N)_{14}$▲...5'		3'...GGGTGT$(N)_{9}$▲...5'
Bsa I	5'...GGTCTC$(N)_{1}$▼...3' 3'...CCAGAG$(N)_{5}$▲...5'	Sap I	5'...GCTCTTC$(N)_{1}$▼...3' 3'...CGAGAAG$(N)_{4}$▲...5'
Bsb I	5'...CAACAC...3' 3'...GTTGTG...5'	UbaF2 I	5'...GAAAY$(N)_{5}$RTG▼...3' 3'...CTTTY'N$)_{5}$R'AC▲...5' **R**=A or G; **Y** =C or T
BseR I	5'...GAGGAG$(N)_{10}$▼...3' 3'...CTCCTC$(N)_{8}$▲...5'	UbaP I	5'...CGAACG▼...3' 3'...GCTTGC$_{7}$▲...5'

B. DNA Polymerase

[0038] DNA polymerases useful in this method include those that are capable of extending from the nick while displacing the down stream strand. Importantly, the polymerase should lack any 5' to 3' exonuclease activity. Useful DNA polymerases are *Bst* DNA Polymerase Large Fragment (New England Biolabs, Ipswich, MA), *Bca* DNA polymerase (Takara Shuzo, Shiga., Japan), Klenow fragment of DNA polymerase I, for example Klenow Fragment, exo-, Phi29 DNA polymerases (FERMENTAS, Hanover, MD), Sequenase 2.0 T7 DNA Polymerase (Amersham Biosciences Corp, Piscataway, NJ), T5 DNA polymerase. Preferred DNA polymerases are *Bst* and *Bca* DNA polymerases.

C. Additional Assay Components

[0039] The reaction mixture also typically comprises appropriate buffers (*e.g.*, phosphate buffers), a source of magnesium ions (*e.g.*, $MgCl_2$), and dNTPs (dATP, dGTP, dCTP and TTP) 50 nM forward primer, 500 nM reverse primer, 50 nM each bumper, 4U Bst DNA polymerase, 4U BbvC1B (New England Biolabs), 0.1U Endo IV (Trevigen) diluted in Diluent A (New England Biolabs). After addition of all above components, including the three enzymes at room temperature, reaction tubes were placed directly in the thermocycler and were incubated at 49°C for 50min.

D. Detection Systems

[0040] The target amplified by the isothermal strand displacement method of the current invention can be detected in numerous ways. In one preferred embodiment, the isothermal strand displacement method is coupled to the endonuclease IV signal amplification method. The endonuclease IV signal amplification method is disclosed in U. S. Patent Publication No. 2003/026133, which is hereby incorporated herein by reference in its entirety. This method requires i) a short FRET probe, with a quencher preferably at the 5'-end and fluorescent dye

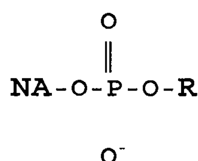
coupled to 3'-hydroxyl through a phosphate bond and rigid linker; ii) a shorter enhancer oligonucleotide and iii) an endonuclease IV enzyme. Both the probe and the enhancer are complementary to the target or amplified target, hybridizing in such a fashion that there is a one base gap between probe and enhancer, generating a substrate for the endonuclease IV,
5 which specifically hydrolyzes the phosphate linkage between the oligonucleotice and the fluorescent dyed (Figure 2). The endonuclease IV does not hydrolyze unhybridized FRET probes. In some embodiments the SDA amplified target is detected with the FRET probe and the endonuclease IV without the enhancer.

[0041] In other embodiments the amplified isothermal target of the invention is detected by
10 a probe that fluoresces on hybridization to its complementary target sequence. Minor groove binder-based probes that fluoresce on hybridization have been disclosed in U.S. Patent Publications Nos. 2003/0175728 and 2005/0214797 which is hereby incorporated herein by reference in its entirety. Molecular beacon probes, containing hairpin-stem regions have been disclosed in U.S. Patent No. 5,312,728. A cleavable hairpin probe was disclosed (Nadeau et
15 al., *Anal. Biochem.*, 276: 177-187 (1999)) to detect SDA amplification product. In some embodiments the isothermally amplified target of the inventions is detected directly or indirectly by labeled or FRET labeled primers.

[0042] In some embodiments, the amplified target of isothermal SDA of this invention is detected by primer-based methods. Primer-based detection methods are either indirect or
20 direct. Indirect detection is performed by using biotinylated primers in a sandwich format utilizing a biotin-straptavidin capture and a detection probe or a variation thereof. Direct primer-based detection typically require a hairpin FRET primer that when hybridized to its complementary extend target strand, fluoresces (U.S. Patent No. 6,656,680).

[0043] In some embodiments, the amplified target of isothermal SDA of this invention is
25 detected using an AP site probe as described in. *e.g.*, U.S. Patent Publication No. 2004/0101893.

[0044] Generally, the structure of an AP site probe is as follows:



[0045] An AP site probe is comprised of a nucleic acid ("NA") covalently bound by its 3′-terminal oxygen atom to a functional, chemical tail ("R") through a phosphodiester group.

[0046] The number of nucleotides in the **NA** component can be 3 to 200, 3 to 100 or 3 to 200 nucleotides in length, depending on the intended use. Usually, the length of the **NA** is from 5 to 30 nucleotides. More typically, the length of the **NA** is 6-25, 7-20, or 8-17 nucleic acids. Most often, the **NA** component is about 6, 7, 8, 9, 10, 11, 12, 13, 14, 15 or 16 nucleic acids in length. Usually, the **NA** component will have a hybridization melting temperature of about 10 to 80°C, more typically of about 20 to 70°C, and preferably about 30°C, 40°C, 50°C or 60°C.

[0047] The sugar, or glycoside, portion of the **NA** component of the conjugates can comprise deoxyribose, ribose, 2-fluororibose, and/or 2-O-alkyl or alkenylribose wherein the alkyl group comprises 1 to 6 carbon atoms and the alkenyl group comprises 2 to 6 carbon atoms. In the naturally-occurring nucleotides, modified nucleotides and nucleotide analogues that can comprise an oligonucleotide, the sugar moiety forms a furanose ring, the glycosidic linkage is of the beta configuration, the purine bases are attached to the sugar moiety via the purine 9-position, the pyrimidines via the pyrimidine 1-position and the pyrazolopyrimidines via the pyrazolopyrimidine 1-position (which is equivalent to the purine 9-position). In a preferred embodiment, the sugar moiety is 2-deoxyribose; however, any sugar moiety known to those of skill in the art that is compatible with the ability of the oligonucleotide portion of the compositions of the invention to hybridize to a target sequence can be used.

[0048] In one preferred embodiment, the **NA** is DNA. An AP site probe comprising DNA can be used to detect DNA, as well as RNA, targets. In another embodiment, the **NA** is RNA. An AP site probe comprising RNA is generally used for the detection of target DNAs. In another embodiment, an AP site probe can contain both DNA and RNA distributed within the probe. In mixed nucleic acid probes, DNA bases preferably are located at 3′-end of the probe while RNA bases are at the 5′-end. It is also preferred when the 3′-terminal nucleotide

is 2′-deoxyribonucleotide (DNA) and when at least four 3′-terminal bases of **NA** are DNA bases.

[0049] Usually, the **NA** component contains the major heterocyclic bases naturally found in nucleic acids (uracil, cytosine, thymine, adenine and guanine). In some embodiments, the
5 **NA** contains nucleotides with modified, synthetic or unnatural bases, incorporated individually or multiply, alone or in combination. Preferably, modified bases increase thermal stability of the probe-target duplex in comparison with probes comprised of only natural bases (i.e., increase the hybridization melting temperature of the probe duplexed with a target sequence). Modified bases include naturally-occurring and synthetic modifications
10 and analogues of the major bases such as, for example, hypoxanthine, 2-aminoadenine, 2-thiouracil, 2-thiothymine, inosine, 5-N^4-ethenocytosine, 4-aminopyrrazolo[3,4-d]pyrimidine and 6-amino-4-hydroxy-[3,4-d]pyrimidine. Any modified nucleotide or nucleotide analogue compatible with hybridization of an AP site probe with a target nucleic acid conjugate to a target sequence is useful in the practice of the invention, even if the
15 modified nucleotide or nucleotide analogue itself does not participate in base-pairing, or has altered base-pairing properties compared to naturally-occurring nucleotides. Examples of modified bases are disclosed in U.S. Patent Nos. 5,824,796; 6,127,121; 5,912,340; and PCT Publications WO 01/38584; WO 01/64958, each of which is hereby incorporated herein by reference in its entirety. Preferred modified bases include 5-hydroxybutynyl uridine for
20 uridine; 4-(4,6-Diamino-1H-pyrazolo[3,4-d]pyrimidin-3-yl)-but-3-yn-1-ol, 4-amino-1H-pyrazolo[3,4-d]pyrimidine, and 4-amino-1H-pyrazolo[3,4-d]pyrimidine for adenine; 5-(4-Hydroxy-but-1-ynyl)-1H-pyrimidine-2,4-dione for thymine; and 6-amino-1H-pyrazolo[3,4-d]pyrimidin-4(5H)-one for guanine. Particularly preferred modified bases are "Super A™," "Super G™: 4-hydroxy-6-amino pyrazolopyrimidine" (www.nanogen.com) and "Super T™".
25 Modified bases preferably support the geometry of a naturally occurring B-DNA duplex. Modified bases can be incorporated into any position or positions in an AP site probe, but preferably are not incorporated as the 3′-terminal base.

[0050] In another embodiment, some or all nucleotides of **NA** are substituted or contain independently different sugar-phosphate backbone modifications including 2'-O-alkyl RNA
30 nucleotides, phosphorothioate internucleotide linkage, methylphosphonate, sulfamate (e.g., U.S. Pat. No. 5,470,967) and polyamide (i.e., peptide nucleic acids, PNA), LNA (locked nucleic acid), and the like. Such modifications and others of potential use in the present invention are described, for example, in Boutorine, *et al.*, *Biochimie* 76:23 (1994); Agrawal,

et al., *Proc. Natl. Acad. Sci.* 88:7595 (1991); Mag, *et al.*, *Nucleic Acids Res.* 19:1437 (1991); Kurreck, *Eur. J. Biochem.* 270:1628 (2003); Lesnik, *et al.*, *Biochemistry* 32:7832 (1993); Sproat, *et al.*, *Nucleic Acids Symp. Ser.* 24:59 (1991); Iribarren, *et al.*, *Proc. Natl. Acad. Sci.* 87:7747 (1990); Demidov, *Trends Biotechnol.* 21:4 (2003); Nielsen, *Methods Mol. Biol.* 208:3 (2002); Nielsen and Egholm, *Curr. Issues Mol. Biol.* 1:89 (1999); Micklefield, *Curr. Med. Chem.* 8:1157 (2001); Braasch, *et al.*, *Chem. Biol.* 8:1 (2001); and Nielsen, *Curr. Opin. Biotechnol.* 12:16 (2001).

[0051] Within the scope of present invention, modifications of the bases and sugar-phosphate backbone as well as other functional moieties conjugated with the probe can serve to improve the sequence specificity of the target-probe duplex formation. In particular, binding between the probe and a matched target nucleic acid is detectably increased over binding to a mismatched target nucleic acid. By "matched target nucleic acid" is intended a target nucleic acid that contains a sequence that is completely complimentary to the probe sequence. By "mismatched target nucleic acid" is intended a polynucleotide that contains a sequence that is partially complimentary to the probe sequence such that it contains at least one mismatched, non-complimentary base, deletion or insertion in comparison to the probe sequence. For example, use of modified bases in an AP site probe allows for more stable base pairs than when using natural bases and enables the use of shorter probes for the same reaction conditions. Reduction of the probe length increases the ability of the probe to discriminate a target polymorphism as small as a Single Nucleotide Polymorphism ("SNP") due to a proportional increase in the contribution of each duplex base pair to the overall duplex stability. In general, the shorter the probe, the greater the relative contribution of an individual base pair in to the overall duplex stability, and the better the probe discrimination of the target polynucleotide polymorphism.

[0052] The functional tail **R** enables detection of the endonuclease tail-cleavage reaction. The structure of **R** can be of any size and composition as long as it supports the template-specific, endonuclease tail-cleavage reaction. **R** can be as large as a natural protein with molecular mass up to 1,000,000 Daltons or it can be as small as a single atom (i.e., a radioactive isotope, such as a hydrogen or an iodine). Since the enzymatic hydrolysis occurs between the 3′-terminal oxygen atom of the **NA** and the phosphorus atom of the phosphodiester bond, for the purposes of the present invention, the phosphate moiety of the probe is considered a part of the functional tail **R**. For example, when **R** is hydrogen (**R** = -H), the functional tail of the probe is a phosphate moiety $-P(O)(OH)_2$ or $-PO_3^{2-}$. The tail **R**

can be hydrophobic or hydrophilic, electrically neutral, positively or negatively charged. It can be comprised of or include independently different functional groups, including mass tags, fluorescent or non-fluorescent dyes, linkers, radioisotopes, functional ligands like biotin, oligopeptides, carbohydrates and the like. For example, as demonstrated herein,
5 Endonuclease IV from *E. coli* efficiently cleaves from the 3′-end of a probe bound to the target nucleic acid a relatively hydrophilic, negatively charged fluorescein moiety as well as an electrically neutral, hydrophobic quenching dye.

[0053] The tail **R** can contain components that improve specificity by blocking non-specific cleavage reactions in the absence of a target molecule without affecting the target-
10 dependent, specific reaction. It is also within the scope of present invention that the tail **R** or some structural components of it can improve the specificity of the target-probe or enhancer-probe complementary binding so that the thermodynamic difference in the probe/enhancer binding to matched and mismatched target nucleic acids is increased. Examples of such structural components are minor groove binders (MBs).

15 **[0054]** The functional tail **R** can incorporate mono-, oligo- or polynucleotides. Nucleotide residues introduced into the tail structure are not intended to bind to the target nucleic acid.

[0055] In addition to a functional chemical tail **R** conjugated to the 3′-end of an AP site probe through a phosphodiester group, the probe optionally can contain other tails and functional moieties covalently attached to the probe or the tail via an appropriate linker.
20 Preferably, the additional moieties do not interfere with endonuclease recognition of the AP tail-cleavage site or the template-specific tail-cleavage reaction. In one embodiment, additional moieties are attached to the 5′-end of the **NA** portion of the probe. In another embodiment, an additional moiety is conjugated to nucleotide bases of the probe such that, when the probe-target duplex is formed, the moieties are located within the major groove of
25 the duplex.

[0056] Incorporation of a moiety in addition to the functional, chemical tail can serve to improve the probe hybridization properties. Examples of such moieties include minor groove binders and intercalators. Minor groove binders are described in U.S. Patent Nos. 6,492,346 and 6,486,308, both of which are hereby incorporated herein by reference. In other
30 embodiments, these moieties operate in conjunction with the functional tail **R** to aid in the detection of an endonuclease tail-cleavage reaction. Examples of such moieties include radioisotopes, radiolabelled molecules, fluorescent molecules or dyes, quenchers (dyes that

quench fluorescence of other fluorescent dyes), fluorescent antibodies, enzymes, or chemiluminescent catalysts. Another suitable moiety is a ligand capable of binding to specific proteins which have been tagged with an enzyme, fluorescent molecule or other detectable molecule (for example, biotin, which binds to avidin or streptavidin, or a hemin
5 molecule, which binds to the apoenzyme portion of catalase).

[0057] In a preferred embodiment, both the functional tail **R** and the additional moiety are dyes. One or both of the tail and additional moiety dyes can be fluorescent dyes. Preferably, one of the dyes is fluorescent. In one preferred embodiment the functional tail comprises a fluorescent dye and the additional moiety comprises a quencher. The fluorescent dye and
10 quencher molecule operate together such that the fluorescence of the dye is repressed when the dye is bound to the AP site probe, but the fluorescence of the dye is detectable when the phosphodiester bond between the **NA** and tail **R** is hydrolyzed or cleaved by the enzyme. This fluorescence detection strategy is known as Fluorescence Resonance Energy Transfer (FRET). According to a FRET technique, one of the dyes servers as a reporter dye and the
15 other dye is a quencher that substantially decreases or eliminates fluorescence of the reporter dye when both of the dyes are bound to the same molecule in proximity of each other. The fluorescence of the reporter dye is detected when released from the proximity of the quencher dye. Cleavage of the AP site probe functional tail releases the reporter dye from its quencher counterpart allowing for a detectable increase in the reporter fluorescence and detection of the
20 target nucleic acids. The quenching dye can be a fluorescent dye or non-fluorescent dye (dark quencher). *See*, U.S. Patent Publication Nos. 2003/0113765 and 2003/0096254 and PCT Publication No. WO 01/42505 for fluorophore and quencher examples, both of which are hereby incorporated herein by reference.

[0058] The present invention includes a composition comprising a solid support and an AP
25 site probe immobilized thereon. In such a case, one of the moieties conjugated to the probe can be a moiety that serves to attach the probe to the solid support. This moiety or solid support linker can be attached anywhere within or be a structural part of the **NA** and functional tail **R** structures of the probe of the present invention. In one embodiment, the AP site probe is covalently attached to a solid support through a Schiff base type linkage, as
30 described in U.S. Patent No. 6,548,652, incorporated herein by reference.

[0059] In assays of the present invention, an AP site probe is typically included at concentrations of about 50-200 nM, more typically at concentrations of about 100-175 nM,

and preferably at concentrations of about 150 nM. One of skill in the art will appreciate that the probe concentrations provided above can be altered depending on a variety of factors, including the amount of target, as well as the characteristics of the dye or quencher used.

[0060] An enhancer is an oligo- or polynucleotide designed to form a duplex with the target
5 nucleic acid positioned immediately 5′- to the target-AP site probe. The combined, probe-enhancer-target complex simulates a naturally occurring nucleic acid atypical abasic site that is recognized by cellular exo- and endonuclease repair enzymes. Although the tail **R** cleavage reaction can be achieved without the enhancer, the presence of an enhancer generally improves the kinetics the reaction. The probe and enhancer form duplexes with the
10 target nucleic acid that are positioned next to each other leaving one, non-paired base of the target between the duplexes. Although this is a preferred design, cleavage of the tail **R** in the target-probe complex can be achieved in absence of the enhancer, or when the number of non-paired, target polynucleotide bases between two duplexes shown is 1, 2, 3, 4, 5 or more bases.

15 **[0061]** The structural requirements and limitations for an enhancer are essentially the same as for a **NA** component of an AP site probe, described above. Generally, the number of nucleotides in an enhancer oligonucleotide can range from 3 to 50, 100 or 200 nucleotides in length. Usually, the length of an enhancer is from 5 to 30 nucleotides. More typically, the length of the enhancer is 6-25, 7-20, or 8-15 nucleic acids. Most often, an enhancer
20 component is about 10, 12, 14, 16, 18 or 20 nucleic acids in length. Usually, an enhancer oligonucleotide component will have a hybridization melting temperature of about 10 to 80°C, more typically of about 20 to 70°C, and preferably about 30°C, 40°C, 50°C, 60°C or 70°C. An enhancer oligonucleotide will usually have a comparatively equal or higher hybridization melting temperature in comparison to the melting temperature of the **NA**
25 component of the AP site probe. Usually, the melting temperature will be about 5 to 30°C, more typically about 10 to 20°C, and preferably about 8°C, 10°C, 15°C, or 20°C higher than the melting temperature of the **NA** component of the AP site probe.

[0062] Preferably, the enhancer is DNA. An oligo- or polydeoxyribonucleotide enhancer is useful for detecting DNA and RNA target nucleic acids. The enhancer can also be RNA. In
30 another embodiment, an enhancer can contain both DNA and RNA. Preferably, DNA bases are located at the 5′-end of the enhancer while RNA bases are at its 3′-end. Preferably, at least the four 5′-terminal bases of the enhancer are DNA bases.

[0063] In another embodiment, the enhancer contains nucleotides with modified, synthetic or unnatural bases, including any modification to the base, sugar or backbone. Preferably, modified bases increase thermal stability of the enhancer-target duplex in comparison to enhancer sequences that contain only natural bases. Specific modified bases are the same as 5 those described for a probe.

[0064] In another embodiment, some or all nucleotides of the enhancer are substituted or contain independently different sugar-phosphate backbone modifications, including, 2'-O-alkyl RNA nucleotide, phosphorotioate internucleotide linkage, PNA (peptide nucleic acid), LNA (locked nucleic acid). References describing these and other potentially useful sugar-10 phosphate backbone modifications are provided above.

[0065] The enhancer optionally can contain some functional tails or markers conjugated to either end of the enhancer or in the middle of it. These moieties should not interfere with the template-specific cleavage of the probe **R** tail. In a preferred embodiment, these moieties are attached to the 3'-end of the enhancer. In another preferred embodiment, these moieties are 15 conjugated to nucleotide bases of the enhancer such that, when the enhancer-target duplex is formed, the moieties are located within the major groove of this duplex. Enhancer moieties can serve to improve the enhancer hybridization properties. Examples of such moieties include minor groove binders and intercalators.

[0066] The present invention also encompasses a composition comprising an enhancer 20 immobilized on a solid support. A moiety conjugated to the enhancer can serve to attach the enhancer to the solid support. This moiety or solid support linker can be attached anywhere within or be a structural part of the enhancer.

[0067] Modifications of the bases and sugar-phosphate backbone as well as other functional moieties conjugated to the enhancer can serve to improve the sequence specificity 25 of target-enhancer duplex formation resulting in increased thermodynamic differences in binding between the enhancer and a matched target nucleic acid in comparison to binding between the enhancer and a mismatched target nucleic acid.

[0068] In assays of the present invention, an enhancer, when included, is typically added at concentrations of about 50-200 nM, more typically at concentrations of about 100-175 nM, 30 and preferably at concentrations of about 150 nM. One of skill in the art will appreciate that the enhancer concentrations provided above can be altered depending on a variety of factors, including the amount of target, as well as the amount of probe used and its characteristics.

[0069] An enzyme used in conjunction with the AP site probe is an endonuclease or exonuclease that recognizes an Apurinic/Apyrimidinic (AP) site or atypical AP site moiety simulated by an AP site probe duplexed with a target nucleic acid complex, and preferentially hydrolyzes or cleaves the phosphodiester bond between the probe and the functional tail **R**.
5 An enhancer can be used to increase the kinetics of the tail-cleavage reaction. An enzyme useful in the present methods preferentially does not cleave the **NA** part of the probe or the target nucleic acid. Otherwise, enzymes which cleave the probe **NA** or target nucleic acid at an efficiency that is substantially lower than target-specific tail cleavage can still find use in practicing the present methods. To minimize non-specific detection of the target nucleic
10 acid, the enzyme preferentially does not cleave the tail **R** of the probe in absence of the target nucleic acid.

[0070] In a preferred embodiment, the enzyme is an AP endonuclease. The enzyme can be a class I or a class II AP endonuclease. Preferably, the enzyme is a class II endonuclease. Enzymes that belong to this family are isolated from variety of organisms, and any class II
15 endonuclease that specifically recognizes an AP abasic site and specifically hydrolyzes the phosphodiester backbone on the 5′ side of the AP site can be used in the present methods. Exemplified class II AP endonucleases include Endonuclease IV and Exonuclease III from *E. coli*, human APE1/REF-1 endonuclease, yeast APN1 endonuclease, exonuclease III homologous enzymes from *Drosophila* (Rrp1) and *Arabidopsis* (Arp) and thermostable
20 endonuclease IV from *Thermotoga maritima*. Other AP endonucleases useful for detection and/or amplification systems requiring an AP site probe can be identified through the National Center for Biotechnological Information Entrez/PubMed nucleotide and protein databases accessed through the website www.ncbi.nlm.nih.gov/. Enzymes homologous in structure and function to the *E. coli* Exonuclease III family of AP nucleases are also of use in
25 the present invention (Mol, *et al.*, *Mutat. Res.* 460:211 (2000); Ramotar, *Biochem. Cell Bio.* 75:327 (1997)). The structure and function of apurinic/apyrimidinic endonucleases is reviewed by Barzilay and Hickson in *Bioessays* 17:713 (1995).

[0071] In a preferred embodiment, the enzyme is an *E. coli* Endonuclease IV. An *E. coli* Endonuclease IV exhibits catalytic activity between room temperature (25°C) and 75°C,
30 preferably between 40-70°C or 40-60°C, and more preferably between 60-70°C or 65-75°C. The temperature of a target nucleic acid detection assay is preferably determined by the hybridization melting temperature of an AP site probe, where the temperature of the reaction conditions is preferably within 5, 4, 3, 2, 1 or 0 degrees, above or below, of the probe melting

temperature, T_m. Optimum catalytic activity of an Endonuclease IV is observed within a pH range of 7.5-9.5, preferably between pH 8.0-9.0, most preferably at about pH 8.5-9.0. An abasic site assay using an Endonuclease IV enzyme is preferably carried out using a buffer that maintains a steady pH value of between 7.5-9.5 over varying temperatures. Preferred
5 buffers include HEPPS (4-(2-hydroxyethyl)-1-piperazinpropan-sulfonic acid) and HEPES (4-(2-Hydroxyethyl)piperazine-1-ethanesulfonic acid). In a preferred embodiment, the buffer used is HEPPS-KOH. In certain embodiments, a TRIS buffer is also appropriate. Additional biological buffers of potential use can be found through Sigma-Aldrich (St. Louis, MO, www.sigma.com). Usually, the reaction conditions contain enzyme in nanomolar
10 concentrations, but tail cleaving activity can be observed when the enzyme is provided in picomolar concentrations, and in certain cases in femtomolar concentrations.

[0072] Either part of the endonuclease tail-cleavage reaction, the **NA** containing part or the tail **R** containing part or alternatively both of them independently, can be detected. Suitable reporter groups for attaching to the functional tail **R** include beads, nanoparticles (Taton, *et*
15 *al.*, *Science* 289:1757 (2000), chemiluminescers, isotopes, enzymes and fluorophores. A variety of physical or chemical methods can be used for detection of the cleavage product. Depending on the nature of the markers used, these methods include, for example, chromatography and electron-, UV-, IR-, mass-, radio-, fluorescence spectroscopy including fluorescence polarization and the like.

20 **[0073]** In a preferred embodiment, cleavage of the functional tail **R** comprises a fluorophore reporter group and is detected by fluorescence spectroscopy. Suitable fluorophores include the resorufin dyes, coumarin dyes, xanthene dyes, cyanine dyes, BODIPY dyes and pyrenes. Preferably, the functional tail **R** comprises a fluorescent dye with a xanthene core structure. Additional fluorophores appropriate for incorporation into the
25 functional tail **R** are described in PCT Publication Nos. WO 01/142505 and WO 06/020947 and in Haugland, *Handbook of Fluorescent Probes and Research Products*, Ninth Ed., (2002), published by Molecular Probes, Eugene, OR (accessible at www.probes.com/handbook/).

[0074] In some embodiments, background fluorescence of a fluorophore incorporated on
30 the functional tail **R**, is minimized by attaching a quencher to the AP site probe. Typically, a quenching molecule is covalently attached to the 5′ end of the probe through a linker that is not cleaved by an enzyme. In some embodiments, a quencher is linked to the middle or the 3′

end of the probe. When a quencher is attached to the 3′ end of the probe, it is usually incorporated into the functional tail **R** as a "cleavable quencher," and the fluorophore is then attached to the middle or the 5′ end of the probe. However, any molecule that neutralizes or masks the fluorescence of a fluorophore incorporated in an uncleaved functional tail **R** finds
5 use as a quencher in the present invention. Other quencher molecules suitable to attach to an AP site probe and guidance for selecting appropriate quencher and fluorophore pairs is provided in Haugland, *supra*. Additional guidance is provided in U.S. Patent Nos. 3,996,345 and 4,351,760, and U.S. Publication Nos. 2003/0096254 and 2003/0113765 and in co-owned U.S. Patent Application No. 09/457,616, filed on December 8, 1999, each of which is hereby
10 incorporated herein by reference.

[0075] Fluorophore and cleavable quencher molecules are typically attached to an AP site probe through a linker that is specifically cleaved by an enzyme. A linker can be rigid or flexible. Preferably the linker structurally mimics a naturally occurring abasic site, and is cleaved by an Endonuclease IV. Preferably the C1 carbon of the linker, attached to the
15 phosphate, is a primary carbon. Preferably the linker comprises a phosphate. Suitable commercially available chemical linkers can be purchased through Pierce Biotechnology, Rockford, IL and Molecular Probes, Eugene, OR. Suitable methods for attaching reporter groups such as fluorophores and quenchers through linkers to oligonucleotides are described in, for example, U.S. Patent Nos. 5,512,677; 5,419,966; 5,696,251; 5,585,481; 5,942,610 and
20 5,736,626, each of which are hereby incorporated herein by reference.

[0076] In a preferred embodiment the linker is a rigid linker. In one preferred embodiment, the rigid linker is a hydroxyprolinol linker. Hydroxyprolinol linkages are described in U.S. Patent Nos. 5,419,966; 5,512,677; 5,519,134; and 5,574,142 each of which is incorporated herein by reference. Cleavage of the functional tail **R** attached through a rigid linker, i.e., a
25 hydroxyprolinol linker, requires greater concentrations of enzyme and exhibits decreased catalytic rates, but is highly specific. Generally, the Endonuclease IV enzyme does not detectably cleave functional tails **R** attached to an AP site probe through a rigid linker, such as a hydroxyprolinol linker, in the absence of a target nucleic acid.

[0077] In some embodiments, it is desirable to attach the functional tail **R** through a
30 flexible linker. Cleavage of the functional tail **R** is more efficient when attached through a flexible linker, however, decreased specificity is observed because detectable tail-cleavage occurs in the absence of a target nucleic acid. Non-specific cleavage of functional tails **R**

attached through a flexible linker can be minimized by adding a competitive binding substrate that is more favorable to the enzyme than an unduplexed probe but less favorable than the probe duplexed with a target nucleic acid, i.e., a "decoy." In one embodiment unmelted genomic DNA is added to the reaction as a decoy to minimize cleavage of the AP site probe functional tail **R** in the absence of a target nucleic acid.

[0078] The ability of particular tail structures to serve as specific substrates of an AP endonuclease can be determined using an assay that provides a probe/target nucleic acid/enhancer complex as a single hairpin structure. Preferably the hairpin structure has one unpaired nucleic acid, thereby simulating a naturally occurring abasic site residing in duplexed nucleic acids. In other embodiments, the test assay hairpin structure can have zero or two unpaired nucleic acids. In such a test assay, the cleavage of the functional tail **R** is detected by measuring the release of the reporter group attached to a hairpin structure in comparison to release of the reporter group attached to an unduplexed AP site probe. A tail structure that serves as a specific substrate for an AP endonuclease will be cleaved from a hairpin structure at a faster catalytic rate in comparison to its cleavage rate from an unduplexed AP site probe. A tail structure that serves as a specific substrate preferably exhibits a ratio of specific cleavage, in the presence of the hairpin structure, to non-specific cleavage, in the presence of an unduplexed AP site probe, of at least 50-, 75-, or 100-fold, more preferably of 300-, 400-, 500-, 600-, 700-, 800-, 900- or 1000-fold, and can exhibit ratios of greater than 1000-fold, as measured by the reporter group signal (i.e., Fluorescence Units per minute of a fluorophore reporter group). In some embodiments, the hairpin substrate design does not incorporate a quencher moiety. Nevertheless AP endonuclease cleavage of the fluorescent tail increases the dye fluorescence by approximately two times. The fluorescent signal outcome of the assay can be improved by incorporation of a quenching moiety within the hairpin sequence that represents an enhancer. Those skilled in the art will appreciate that the hairpin substrate can be used for detection as well as for quantitative measurement of AP endonuclease activity in different media.

[0079] In other embodiments, the **NA** part of the AP site probe is detected. For instance, the products of the probe tail-cleavage reaction can be detected as a result of another reaction that follows the cleavage reaction or occurs simultaneously with it. Cleavage of the tail **R** from the probe generates a "free" 3′-hydroxyl group that can be, for example, extended by a polymerase in a template-dependent polynucleotide synthesis in the presence of NTPs such that the tail-OFF probe would serve as a primer complexed with template. In some

embodiments, the strands of a probe extension nucleotide synthesis are the detectable reaction product. Some NTPs incorporated in a probe extension can optionally carry a detectable marker. Incorporation of one or more detectable markers into a probe extension product simplifies the detection of the synthesized nucleotide strands.

5 **[0080]** In one embodiment, a probe is linked to an enhancer so as these two components of the reaction complex are associated with each other during the tail cleaving reaction. The linker can be a covalent or a non-covalent linker, i.e., when interaction between a probe and enhancer is provided by hydrogen bonds or Van der Waals forces. A probe-enhancer linker can be attached at any position within the probe and enhancer. Preferably, the linker does not 10 block the tail cleaving reaction, and is of an appropriate length to support the tail cleaving reaction. Further, a linker useful in a tail cleaving assay will not compromise the ability of the AP site probe or enhancer to form duplexes with a target nucleic acid. Finally, a preferred linker is not cleaved by an AP endonuclease. When attached through a linker, the probe and enhancer are components of one molecule or complex. Linked probe-enhancer 15 molecules or complexes can be immobilized on a solid support.

[0081] In preferred embodiments, a probe-enhancer linker is comprised of individual or combined repeats of substituted alkyl backbone moieties, including $(-OCH_2CH_2-)_n$, $(-OCH_2CH_2-OPO_2-)_n$ or $-O(CH_2)_nO-$. Typically, n is from 1-100, more typically n is 10, 20, 40, 50, 60 or 80. In other embodiments, a linker is a flexible polypeptide chain, for instance, 20 dihydropyrroloindole peptides or a series of one or more repeats of a Gly-$(Ser)_4$ polypeptide sequence. In another embodiment, the linker is an oligonucleotide, such as poly A or poly T and the like. In yet another embodiment, the linker is an alkyl chain having a backbone typically of about 100, 200 or 300 atoms, more typically of about 40, 60 or 80 atoms. Other alkyl linkers of potential use are described in U.S. Patent Publication No. 2003/0113765, 25 incorporated herein by reference. Additional linkers that may find use are described by Dempey, *et al.*, *Nucleic Acids Res.* 27:2931 (1999); Lukhtanov, *et al.*, *Nucleic Acids Res.* 25:5077 (1997); Lukhtanov, *et al.*, *Bioconjug. Chem.* 7:564 (1996); and Lukhtanov, *et al.*, *Bioconjug. Chem.* 6:418 (1995). Appropriate linkers can be obtained from commercially available sources, for example from Pierce Biotechnology, Rockford IL. Guidance for 30 selecting an appropriate linker for attaching oligonucleotides is provided in Haugland, *Handbook of Fluorescent Probes and Research Products, supra.* These linkers also find application in attaching an AP site probe or an enhancer to a solid support.

E. Oligonucleotides and Modified Oligonucleotides

[0082] The terms oligonucleotide, polynucleotide and nucleic acid are used interchangeably to refer to single- or double-stranded polymers of DNA or RNA (or both) including polymers containing modified or non-naturally-occurring nucleotides, or to any other type of polymer capable of stable base-pairing to DNA or RNA including, but not limited to, peptide nucleic acids which are disclosed by Nielsen et al. *Science* 254:1497-1500 (1991); bicyclo DNA oligomers (Bolli et al., *Nucleic Acids Res.* 24:4660-4667 (1996)) and related structures.

[0083] The oligonucleotides of the present invention are generally prepared using solid phase methods known to those of skill in the art. In general, the starting materials are commercially available, or can be prepared in a straightforward manner from commercially available starting materials, using suitable functional group manipulations as described in, for example, March, et al., ADVANCED ORGANIC CHEMISTRY – Reactions, Mechanisms and Structures, 4th ed., John Wiley & Sons, New York, NY, (1992).

[0084] The oligonucleotides of the invention can comprise any naturally occurring nucleotides, non-naturally occurring nucleotides, or modified nucleotides known in the art.

[0085] The oligonucleotide primers and probes of the present invention can include the substitution of one or more naturally occurring nucleotide bases within the oligomer with one or more non-naturally occurring nucleotide bases or modified nucleotide bases so long as the primer can initiate amplification of a target nucleic acid sequence in the presence of a polymerase enzyme.

[0086] For example, the oligonucleotide primers may also comprise one or more modified bases, in addition to the naturally-occurring bases adenine, cytosine, guanine, thymine and uracil. Modified bases are considered to be those that differ from the naturally-occurring bases by addition or deletion of one or more functional groups, differences in the heterocyclic ring structure (i.e., substitution of carbon for a heteroatom, or *vice versa*), and/or attachment of one or more linker arm structures to the base. Preferred modified nucleotides are those based on a pyrimidine structure or a purine structure, for example, 7 deazapurines and their derivatives and pyrazolopyrimidines (described in, for example, WO 90/14353 and U.S. Patent No. 6,127,121).

[0087] Exemplified modified bases for use in the present invention include the guanine analogue 6 amino-1*H*-pyrazolo[3,4-d]pyrimidin-4(5*H*)-one (ppG or PPG, also Super G) and the adenine analogue 4-amino-1*H*-pyrazolo[3,4-d]pyrimidine (ppA or PPA). The xanthene analogue 1*H*-pyrazolo[5,4-d]pyrimidin-4(5*H*)-6(7*H*)-dione (ppX) can also be used. These
5 base analogues, when present in an oligonucleotide, strengthen hybridization and improve mismatch discrimination. All tautomeric forms of naturally-occurring bases, modified bases and base analogues may be included in the oligonucleotide conjugates of the invention. Other modified bases useful in the present invention include 6-amino-3-prop-1-ynyl-5-hydropyrazolo[3,4-d]pyrimidine-4-one, PPPG; 6-amino-3-(3-hydroxyprop-1-yny)l-5-
10 hydropyrazolo[3,4-d]pyrimidine-4-one, HOPPPG; 6-amino-3-(3-aminoprop-1-ynyl)-5-hydropyrazolo[3,4-d]pyrimidine-4-one, NH_2PPPG; 4-amino-3-(prop-1-ynyl)pyrazolo[3,4-d]pyrimidine, PPPA; 4-amino-3-(3-hydroxyprop-1-ynyl)pyrazolo[3,4-d]pyrimidine, HOPPPA; 4-amino-3-(3-aminoprop-1-ynyl)pyrazolo[3,4-d]pyrimidine, NH_2PPPA; 3-prop-1-ynylpyrazolo[3,4-d]pyrimidine-4,6-diamino, $(NH_2)_2$PPPA; 2-(4,6-diaminopyrazolo[3,4-
15 d]pyrimidin-3-yl)ethyn-1-ol, $(NH_2)_2$PPPAOH; 3-(2-aminoethynyl)pyrazolo[3,4-d]pyrimidine-4,6-diamine, $(NH_2)_2$PPPANH_2; 5-prop-1-ynyl-1,3-dihydropyrimidine-2,4-dione, PU; 5-(3-hydroxyprop-1-ynyl)-1,3-dihydropyrimidine-2,4-dione, HOPU; 6-amino-5-prop-1-ynyl-3-dihydropyrimidine-2-one, PC; 6-amino-5-(3-hydroxyprop-1-yny)-l,3-dihydropyrimidine-2-one, HOPC; and 6-amino-5-(3-aminoprop-1-yny)-1,3-
20 dihydropyrimidine-2-one, NH_2PC; 5-[4-amino-3-(3-methoxyprop-1-ynyl)pyrazol[3,4-d]pyrimidinyl]-2-(hydroxymethyl)oxolan-3-ol, CH3OPPPA; 6-amino-1-[4-hydroxy-5-(hydroxymethyl)oxolan-2-yl]-3-(3-methoxyprop-1-ynyl)-5-hydropyrazolo[3,4-d]pyrimidin-4-one, CH3OPPPG; 4,(4,6-Diamino-1H-pyrazolo[3,4-d]pyrimidin-3-yl)-but-3-yn-1-ol, Super A; 6-Amino-3-(4-hydroxy-but-1-ynyl)-1,5-dihydro-pyrazolo[3,4-d]pyrimidin-4-one; 5-(4-
25 hydroxy-but-1-ynyl)-1H-pyrimidine-2,4-dione, Super T; 3-iodo-1H-pyrazolo[3,4-d]pyrimidine-4,6-diamine ($(NH_2)_2$PPAI); 3-bromo-1H-pyrazolo[3,4-d]pyrimidine-4,6-diamine ($(NH_2)_2$PPABr); 3-chloro-1H-pyrazolo[3,4-d]pyrimidine-4,6-diamine ($(NH_2)_2$PPACl); 3-Iodo-1H-pyrazolo[3,4-d]pyrimidin-4-ylamine (PPAI); 3-Bromo-1H-pyrazolo[3,4-d]pyrimidin-4-ylamine (PPABr); and 3-chloro-1H-pyrazolo[3,4-d]pyrimidin-4-
30 ylamine (PPACl).

[0088] In addition to the modified bases noted above, the oligonucleotides of the invention can have a backbone of sugar or glycosidic moieties, preferably 2-deoxyribofuranosides wherein all internucleotide linkages are the naturally occurring phosphodiester linkages. In

alternative embodiments however, the 2-deoxy- β-D-ribofuranose groups are replaced with other sugars, for example, β-D-ribofuranose. In addition, β-D-ribofuranose may be present wherein the 2-OH of the ribose moiety is alkylated with a C_{1-6} alkyl group (2-(O-C_{1-6} alkyl) ribose) or with a C_{2-6} alkenyl group (2-(O-C_{2-6} alkenyl) ribose), or is replaced by a fluoro
5 group (2-fluororibose). Related oligomer-forming sugars useful in the present invention are those that are "locked", i.e., contain a methylene bridge between C-4' and an oxygen atom at C-2'. Other sugar moieties compatible with hybridization of the oligonucleotide can also be used, and are known to those of skill in the art, including, but not limited to, α-D-arabinofuranosides, α-2'-deoxyribofuranosides or 2',3'-dideoxy-3'-aminoribofuranosides.
10 Oligonucleotides containing α-D-arabinofuranosides can be prepared as described in U.S. Patent No. 5,177,196. Oligonucleotides containing 2',3'-dideoxy-3'-aminoribofuranosides are described in Chen et al. *Nucleic Acids Res.* **23**:2661-2668 (1995). Synthetic procedures for locked nucleic acids (Singh et al, *Chem. Comm.*, 455-456 (1998); Wengel J., *Acc. Chem. Res.*, **32**:301-310 (1998)) and oligonucleotides containing 2'-halogen-2'-
15 deoxyribofuranosides (Palissa et al., *Z. Chem.*, **27**:216 (1987)) have also been described. The phosphate backbone of the modified oligonucleotides described herein can also be modified so that the oligonucleotides contain phosphorothioate linkages and/or methylphosphonates and/or phosphoroamidates (Chen et al., *Nucl. Acids Res.*, 23:2662-2668 (1995)). Combinations of oligonucleotide linkages are also within the scope of the present invention.
20 Still other backbone modifications are known to those of skill in the art.

[0089] The ability to design probes and primers in a predictable manner using an algorithm, that can direct the use or incorporation of modified bases, minor groove binders, fluorophores and/or quenchers, based on their thermodynamic properties have been described in co pending application Ser. No. 10/032,307, filed December 21, 2001. Accordingly, the use of
25 any combination of normal bases, unsubstituted pyrazolo[3,4-d]pyrimidine bases (e.g., PPG and PPA), 3-substituted pyrazolo[3,4-d]pyrimidines, modified purine, modified pyrimidine, 5-substituted pyrimidines, universal bases, sugar modification, backbone modification or a minor groove binder to balance the T_m (e.g., within about 5-8°C) of a hybridized product with a modified nucleic acid is contemplated by the present invention.

30 **F. Quenchers**

[0090] Recently developed detection methods employ the process of fluorescence resonance energy transfer (FRET) for the detection of probe hybridization rather than direct

detection of fluorescence intensity. In this type of assay, FRET occurs between a donor fluorophore (reporter) and an acceptor molecule (quencher) when the absorption spectrum of the quencher molecule overlaps with the emission spectrum of the donor fluorophore and the two molecules are in close proximity. The excited-state energy of the donor fluorophore is
5 transferred to the neighboring acceptor by a resonance dipole-induced dipole interaction, which results in quenching of the donor fluorescence. If the acceptor molecule is a fluorophore, its fluorescence may sometimes be increased. The efficiency of the energy transfer between the donor and acceptor molecules is highly dependent on distance between the molecules. Equations describing this relationship are known. The Forster distance (R_o) is
10 described as the distance between the donor and acceptor molecules where the energy transfer is 50% efficient. Other mechanisms of fluorescence quenching are also known, such as, collisional and charge transfer quenching. There is extensive guidance in the art for selecting quencher and fluorophore pairs and their attachment to oligonucleotides (Haugland, R.P., HANDBOOK OF FLUORESCENT PROBES AND RESEARCH CHEMICALS, Sixth
15 Edition, Molecular Probes, Eugene, OR, 1996; U.S. Patent Nos. 3,996,345 and 4,351,760 and the like). Preferred quenchers are described in co-owned U.S. Patent Nos 6,727,356 and 6,790,945 and incorporated herein by reference. Additional structures (e.g., mono- and bis-azo dyes) with different combinations of substituents at various positions can be prepared based on compounds and methods known in the dye chemistry field (summarized in the
20 Color Index, Issue 3 on CDD-ROM, pages 4009-4324; Society of Dyers and Colourists, Bradford, England; http://www.sdc.org.uk; and see also WO 01/86001).

[0091] The quenchers disclosed above cover the range from about 400 – 800 nm, and many demonstrate improved quenching when attached to a MGB. While the modified versions illustrate $-N(CH_2CH_2OH)_2$ as a preferred linking group to be used to couple the quencher to
25 oligonucleotides, MGB or solid support, examples of other suitable linkers are known in the art or are provided herein.

[0092] Preferred quenchers for each of the aspects of the invention herein are selected from those disclosed above, as well as bis azo quenchers from Biosearch Technologies, Inc. (provided as Black Hole™ Quenchers: BH-1, BH-2 and BH-3), Dabcyl, TAMRA and
30 carboxytetramethyl rhodamine

G. Fluorophores

[0093] Fluorophores useful in the present invention are generally fluorescent organic dyes that have been derivatized for attachment to the terminal 5' carbon of the oligonucleotide probe, preferably via a linking group. One of skill in the art will appreciate that suitable fluorophores are selected in combination with a quencher which is typically also an organic dye, which may or may not be fluorescent.

[0094] There is a great deal of practical guidance available in the literature for selecting appropriate fluorophore-quencher pairs for particular probes. See, for example, Clegg (cited above); Wu et al. (cited above); Pesce et al., editors, FLUORESCENCE SPECTROSCOPY (Marcel Dekker, New York, 1971); White et al., FLUORESCENCE ANALYSIS: A PRACTICAL APPROACH (Marcel Dekker, New York, 1970); and the like. The literature also includes references providing exhaustive lists of fluorescent and chromogenic (quenching) molecules and their relevant optical properties for choosing fluorophore-quencher pairs, e.g., Berlman, HANDBOOK OF FLUORESCENCE SPECTRA OF AROMATIC MOLECULES, 2ND EDITION (Academic Press, New York, 1971); Griffiths, COLOUR AND CONSTITUTION OF ORGANIC MOLECULES (Academic Press, New York, 1976); Bishop, editor, INDICATORS (Pergamon Press, Oxford, 1972); Haugland, HANDBOOK OF FLUORESCENT PROBES AND RESEARCH CHEMICALS (Molecular Probes, Eugene, 1992); Pringsheim, FLUORESCENCE AND PHOSPHORESCENCE (Interscience Publishers, New York, 1949); and the like. Additionally, methods for derivatizing fluorophores and quenchers for covalent attachment via common reactive groups are also well known. See, for example, Haugland (cited above); Ullman et al., U.S. Pat. No. 3,996,345; Khanna et al., U.S. Pat. No. 4,351,760; and the like.

[0095] Phosphonylated dyes disclosed in co-owned U.S. Patent Application No. 11/202,635 are particularly preferred and includes xanthene-, cyanine- , coumarin-, phenoxazine-,Bodipy-based fluorophores. Other preferred fluorophores are those based on xanthene dyes, a variety of which are available commercially with substituents useful for attachment of either a linking group or for direct attachment to an oligonucleotide. Another group of fluorescent compounds are the naphthylamines, having an amino group in the α- or β-position. Included among such naphthylamino compounds are 1-dimethylaminonaphthyl-5-sulfonate, 1-anilino-8-naphthalene sulfonate and 2-*p*-toluidinyl-6-naphthalene sulfonate. Other dyes include 3-phenyl-7-isocyanatocoumarin, acridines, such as 9-

isothiocyanatoacridine and acridine orange; N-(p-(2-benzoxazolyl)phenyl)maleimide; benzoxadiazoles, stilbenes, pyrenes, and the like. Still other suitable fluorophores include the resorufin dyes, rhodamine dyes, cyanine dyes and BODIPY dyes.

[0096] These dyes and appropriate linking methodologies for attachment to oligonucleotides are described in many references, e.g., Khanna et al. (cited above); Marshall, *Histochemical J.*, 7:299-303 (1975); Menchen et al., U.S. Pat. No. 5,188,934; Menchen et al., European Patent Application 87310256.0; and Bergot et al., International Application No. PCT/US90/05565.

[0097] More particularly, the fluorophores described herein can be attached to the oligonucleotide portions using, for example, chemical or enzymatic methods. By way of example, methods for incorporation of reactive chemical groups into oligonucleotides, at specific sites, are well-known to those of skill in the art. Oligonucleotides containing a reactive chemical group, located at a specific site, can be combined with a label attached to a complementary reactive group (*e.g.*, an oligonucleotide containing a nucleophilic reactive group can be reacted with a label attached to an electrophilic reactive group) to couple a label to a probe by chemical techniques. Exemplary labels and methods for attachment of a label to an oligonucleotide are described, for example, in U.S. Patent No. 5,824,796; U.S. Patent No. 5,210,015; Kessler (ed.), *Nonradioactive Labeling and Detection of Biomolecules*, Springer-Verlag, Berlin, 1992; Kricka (ed.) *Nonisotopic DNA Probe Techniques*, Academic Press, San Diego, 1992; Howard (ed.) *Methods in Nonradioactive Detection*, Appleton & Lange, Norwalk, 1993. Non-specific chemical labeling of an oligonucleotide can be achieved by combining the oligonucleotide with a chemical that reacts, for example, with a particular functional group of a nucleotide base, and simultaneously or subsequently reacting the oligonucleotide with a label. See, for example, Draper *et al.* (1980) *Biochemistry* **19**:1774-1781. Enzymatic incorporation of label into an oligonucleotide can be achieved by conducting enzymatic modification or polymerization of an oligonucleotide using labeled precursors, or by enzymatically adding label to an already-existing oligonucleotide. See, for example, U.S. Patent No. 5,449,767. Examples of modifying enzymes include, but are not limited to, DNA polymerases, reverse transcriptases, RNA polymerases, *etc.* Examples of enzymes which are able to add a label to an already-existing oligonucleotide include, but are not limited to, kinases, terminal transferases, ligases, glycosylases, *etc.*

[0098] For each of the aspects of the present invention, preferred fluorophores are selected from xanthenes, cyanines, BODIPY analogs, 5-FAM, 6-FAM, TET™, JOE™, HEX™, VIC™, NED™, TAMRA™, ROX™, Bothell Blue™ , Gig Harbor Green ™ and Yakima Yellow™. These fluorophores are generally available from commercial sources such as
5 Applied Biosystems Inc., Foster City, CA and Epoch Biosciences, Inc., Bothell, WA.

IV. Kits

[0099] The invention further provides kits comprising components for carrying out the methods described herein. For example, a kit may comprise one container that holds a nicking enzyme (*e.g.*, N.BbvCI), another container that holds an extension primer, another
10 container that holds a bumper primer, another container that holds an extension primer, another container that holds an AP site probe, another container that holds an AP endonuclease , another container that holds an enhancer, and combinations of thereof.

EXAMPLES

[0100] The following examples are provided to illustrate, but to limit the presently claimed
15 invention.

Example 1.

[0101] This example demonstrates the isothermal generation of ssDNA by strand displacement utilizing a nicking enzyme and a polymerase in a SDA amplification reaction and fluorescent detection of amplified target with endonuclease IV signal detection system in
20 a homogenous reaction.

Assay design and oligonucleotide component structures for *Mycobacterium* tuberculosis detection

[0102] A fragment of the *Mycobacterium tuberculosis* IS6110 sequence (GenBank X52471) is shown in Sequence 1. The endogenous N.BbvC1B recognition site is shown in
25 bold and underlined. The locations of the complementary target specific sequences of the amplification primers are shown in bold italics, while the locations of the bumper sequences are shown in lower case. The probe sequence for the endonuclease IV assay is underlined.

Sequence 1.

GAGA**CCTCAGC**CGGCGGCTGGTCTCTGGCGTTGAGCGTAGTAGGCAGCCTCGAGTTCGACCG
30 GCGGGACGTCGCCGCAGTACTGGTAGAGGCGGCGATGGTTGAACCAGTCGACCCAGCGCGCG

GTGGCCAACTCGACATCCTCGATGGACCGCCAGGGCTTGCCGGGTTTGATCAGCTCGGTCTT
GTATAGGCCGTTGATCGTCTCGGCTAGTGCATTGTCATAGGAGCTTCCGACCGCTCCGACCG
ACGGTTGGATGCCTGCCTCGGCGAGCCGCTcgctgaaccggatCGATGTGT***ACTGAGATCCC***
CTATCCGTATGGTGGATAACGTCTTTCA***GGTCGAGTAC***GCCTTCTTgttggcgggtccaGAT
5 GGCTTGCTCGATCGCGTCGAGGACCATGGAGGTGGCCATCGTGGAAGCGACCCGCCAG

Oligonucleotide Sequences

Sequence 2. Forward primer: GCATTATAGTACCTGTCT**CCTCAGC*****ACTGAGATCCCCT***

Sequence 3. Reverse primer: TTGAATAGTCGGTTACTT**CCTCAGC*****GCGTACTCGACC***

Sequence 4. Forward bumper: cgctgaaccggat

10 Sequence 5. Reverse bumper: tggacccgccaac

Sequence 6. Probe: Q - TCCGTA*TGGTG – Fl where Q is a quencher and in this example the Eclipse Dark Quencher and Fl is a fluorophore and in this example Gig Harbor Green™ fluorescent dye. A* is Super A™ modified base.

DNA sample

15 **[0103]** Genomic DNA from *M. tuberculosis* strain SBRI10.

Homogeneous isothermal SDA

[0104] Amplifications were performed on samples containing *M. tuberculosis* target DNA (from strain SBRI10) in 10 µl final volume in a Rotor-Gene 3000 thermocycler (Corbett Research). Each sample contained 36 mM K_2HPO_4, pH7.6, 3.75 mM $MgCl_2$, 0.25 mM each
20 dNTPs (dATP, dGTP, dCTP and TTP), 10ng of human genomic DNA, 50 nM forward primer, 500 nM reverse primer, 50 nM each bumper, 4U Bst DNA polymerase, 4U BbvC1B (New England Biolabs), 0.1U Endo IV (Trevigen) diluted in Diluent A (New England Biolabs). After addition of all above components, including the three enzymes at room temperature, reaction tubes were placed directly in the thermocycler and were incubated at
25 49°C for 50min. Fluorescent readings were taken at one minute intervals in the FAM channel with an excitation and emission wavelengths of 470 and 510 nm, respectively.

[0105] Amplification of no-template control, 20, 200 and 2000 copies of target is shown in Figure 3. As shown amplification occurs rapidly and 20 copies could be determined within 30 minutes.

Example 2.

[0106] This example illustrates the SDA amplification of Factor V Leiden and the subsequent detection of the amplified nucleic acid on a NanoXhip ® microarray (Nanogen , La Jolla, CA). Factor V Leiden (sometimes Factor V Leiden) is a hypercoagulability disorder in which Factor V, one of the coagulation factors, cannot be deactivated. Factor V Leiden is the most common hereditary hypercoagulability clotting disorder amongst Eurasians, possibly affecting up to 5% of the population of the U.S. It is named after the city Leiden (The Netherlands), where it was first identified in 1994 (Bertina et al, Nature 369: 64-67 (1994)).

[0107] Table 1. lists the oligonucleotides used in this example. The amplifiable primers (AP) contain a recognition sequence *CCTCAGC* (underlined) for *N.BbvC*1B. The bumper primer does not contain the recognition sequence. A nest and biotinylated primer (biotin-primer) was included in SDA reaction to allow post-amplification product analysis on NanoChip® platform (the biotin-primers convert the typical non-biotinylated SDA product to biotinylated product to allow anchorage of the SDA product on NanoChip® microarray).

Table 1 Oligonucleotide sequences

Oligo name	Sequence (5' → 3')
Primers:	
FV forward AP	5'-CATCATGAGAGACATCGCCT*CCTCAGC*AATAGGACTAC-3'
FV reverse AP	5'-AAATTCTCAGAATTTCTGAA*CCTCAGC*TTCAAGGACAA-3'
FV reverse bumper	5'-GCCCCATTATTTAGCCAGGA-3'
FV nest primer	5'-bio-TGTAAGAGCAGATCCCTGGAC-3'
Reporters:	
FV Wt disc	5'-CTGAGTCCGAACATTGAGTCCTGTATTCCTCG-3'
FV Mut disc	5'-GCAGTATATCGCTTGACATCCTGTATTCCTTG-3'
FV stab	5' CCTGTCCAGGGATCTGCTCTTAC 3'
WT univ rep probe	5'-CTCAATGTTCGGACTCAG-A532
MUT univ rep probe	5'-TGTCAAGCGATATACTGC-A647

The underlined sequence indicates the nicking recognition sequence; bio is biotin, A532 (green dye) and A647 (red dye) are Alexa Fluors (Invitrogen, Eugene, OR);

SDA of Factor V human (FV) gDNA

[0108] Sample amplification was carried out in a 10μL volume SDA reaction that contained 50ng of gDNA (extracted from human whole blood), 250nM forward and reverse amplifiable primer, 25nM reverse bumper and 500nM biotin-primer, 3.75mM $MgCl_2$, 36mM

K_2HPO_4, pH7.6, 0.25mM each dNTPs (dATP, dGTP, dCTP and dTTP), 4U *N.BbvC*1B and 4U *Bst* DNA polymerase diluted in Diluent A (New England Biolabs, Beverly, MA, USA). All components, including the 2 enzymes, were added together to a 200-μL microcentrifuge tube, either on ice or at room temperature. After a gentle vortex and spin of the reaction mix, 5 the tube was placed on a thermal cycler or a heat block set at 50°C and allowed for 30 min incubation.

Detection of SDA amplified Factor V DNA on a NanoChip® microarray

[0109] Analysis of the post-amplification product was carried out on a NanoChip® microarray. After 30 min incubation, one microliter of the SDA reaction was added to 59μL 10 50mM histidine (60 folds dilution) and electronically addressed on the Nanogen Molecular Biology WorkStation (MBW) Loader to a NanoChip® electronic microarray (Nanogen, La Jolla, CA) where the biotin-products in the SDA reaction would attach to the streptavidin molecules embedded in the permeation layer on the microarray while non-biotin products (including complementary strands) would be washed off the microarray. The microarray was 15 then incubated with a reporter mix containing a stabilizer oligonucleotide, 2 discriminator oligonucleotides and 2 fluorescence labeled oligonucleotide probes for the FV wild type and mutant SNP products, respectively, and scanned on a MBW Reader. The fluorescent signal level detected on the microarray represents the yield of target product (specific to each probe) while the ratio of the 2 fluorescent signals determines the genotype of the gDNA sample. A 20 green:red ratio >5:1 indicates a wild type sample, a green:red ratio <5:1 is for homologous mutant sample while a ratio of ~1:1 is for heterogyzous sample. Figure 4 shows analysis result of 9 gDNA samples that were amplified by the 1-step SDA and analyzed on a NanoChip® microarray.

Example 3.

25 **[0110]** This example illustrates the real-time SDA amplification of Factor V Leiden from human DNA detecting the amplified target with a mutant probe (5'-MGB-Q-CatAaGGA**A**CGGA-FAM-3') and a wild-type probe 5'-MGB-Q-CatAaGGA**G**CGGA-TET-3' where MGB is the minor groove binder ligand, Q is Eclipse Dark Quencher, FAM is fluorescein, TET is tetrachloro-6-carboxyfluorescein (Glen Research, Stirling, VA), "a" and 30 "t" are Super A and Super T; and the bold and underlined letter indicates the SNP base.

Real time 1-step SDA for human gDNA genotype analysis

[0111] Real time genotyping analysis was successfully incorporated into the 1-step SDA. The reaction was run in 0.1mL Strip Tubes (Corbett Robotics, Australia) in a 10μL volume reaction that had a similar composition to the above SDA reaction (but did not contain the
5 nest biotin-primer) and contained 1x dilution of the MGB Eclipse probe mix for the human FV SNP (the probes were designed and manufactured by Nanogen Bothell and the real time probe mix contains two labeled probes, one for FV wild type and one for FV mutant product, both incorporated with the Eclipse™ Dark Quencher, the MGB™ technology and modified Super bases). The reactions, prepared at room temperature, were incubated on a Rotor-Gene
10 3000™ Four-Channel Multiplexing System (Corbett Robotics, Australia) set at 45°C and fluorescent signals were collected every 20 seconds during incubation. Figure 5 shows the real time fluorescent signals from 4 SDA reactions each contained a wild type, a mutant, a heterogyzous gDNA, respectively (one no template control). All real time signals were analyzed by the RG-3000™ software for allele discrimination.

15 [0112] One of ordinary skill in the art will recognize from the provided description, figures, and examples, that modifications and changes can be made to the various embodiments of the invention without departing from the scope of the invention defined by the following claims and their equivalents. Additionally, all references, patents, patent publications and the like are expressly incorporated herein by reference in their entirety for all purposes.

20

WHAT IS CLAIMED IS:

1 1. A method for generating a target nucleic acid sequence for
2 amplification, said method comprising:
3 (a) providing a double stranded target sequence;
4 (b) nicking one strand of said target sequence with a nicking enzyme,
5 thereby generating the target nucleic acid sequence without thermal
6 denaturation of the double stranded target sequence, wherein the recognition site of the
7 nicking enzyme:
8 (i) is at least 6 nucleotides in length,
9 (ii) is present in one strand of the target sequence about 1 to about 50
10 times, or
11 (iii) comprises a combination of (i) and (ii).

1 2. The method of claim 1, wherein the recognition site of the nicking
2 enzyme is at least 6 nucleotides in length.

1 3. The method of claim 1, wherein the recognition site of the nicking
2 enzyme is about 7 to about 14 nucleotides in length.

1 4. The method of claim 3, wherein the recognition site of the nicking
2 enzyme is at least 7 nucleotides in length.

1 5. The method of claim 1, wherein the recognition site of the nicking
2 enzyme is present in one strand of the target sequence about 1 to about 50.

1 6. The method of claim 5, wherein the recognition site of the nicking
2 enzyme is present in one strand of the target sequence about 9 times.

1 7. The method of claim 1, wherein the recognition site of the nicking
2 enzyme is at least 7 nucleotides in length and is present in one strand of the target sequence
3 about 9 times.

1 8. The method of claim 1, wherein the nicking enzyme is a type IIS
2 nicking enzyme.

1 9. The method of claim 8, wherein the nicking enzyme is a modified type
2 IIS nicking enzyme.

1 10. The method of claim 8, wherein the nicking enzyme is a member
2 selected from the group consisting of: Nt.BbvCI, Nb.BsmI, N. BbvC IA, N.BbvC IB,
3 N.BstNB I, N.Alw I, Nb.Bpu101, N.Bst9I, NMlyI, R.BbvCI, Nb.SapI-1 (variant 33) and
4 Nb.SapI-1 (E250K).

1 11. The method of claim 8, wherein the nicking enzyme is Nt.BbvCI.

2 12. A method for amplifying a target nucleic acid sequence, said method
3 comprising:
4 (a) generating a target nucleic acid sequence according to the method of claim
5 1;
6 (b) contacting a first extension primer and a first bumper primer with the
7 target nucleic acid sequence under conditions sufficient to allow first extension primer to
8 hybridize to the target nucleic acid sequence and for the first bumper primer to hybridize to
9 the target nucleic sequence at a site 5' to the binding site of the first extension primer,
10 wherein the 3' end of the first extension primer comprises a target binding
11 sequence and the 5' end of the first extension primer comprises:
12 (i) a recognition sequence for the nicking enzyme and
13 (ii) a sequence which is complementary to the target nucleic acid,
14 (c) simultaneously extending the first extension primer and the first bumper
15 primer with a polymerase to produce a first extension product and a first bumper extension
16 product that displaces the first extension product;
17 (d) contacting a second extension primer and a second bumper primer with the
18 displaced first extension product under conditions sufficient to allow the second extension
19 primer to hybridize to the first extension product and for the second bumper primer to
20 hybridize to the first extension product at a site 5' to the binding site of the second extension
21 primer,
22 wherein the 3' end of the second extension primer comprises a sequence that
23 binds to the first extension product and the 5' end of the second extension primer comprises:
24 (i) a recognition sequence for the nicking enzyme and
25 (ii) a sequence which is complementary to the target nucleic acid; and

26 (e) simultaneously extending the second extension primer and the second
27 bumper primer with the polymerase to produce a second extension product and a second
28 bumper extension product that displaces the second extension product, thereby generating an
29 amplified target sequence.

1 13. The method of claim 12, wherein the polymerase is a DNA polymerase
2 without 5'→3' exonuclease activity.

1 14. The method of claim 13, wherein the polymerase is a member selected
2 from the group consisting of: Bst DNA Polymerase Large Fragment, Bca DNA polymerase,
3 Klenow fragment of DNA polymerase I, Phi29 DNA polymerases, Sequenase 2.0 T7 DNA
4 Polymerase and T5 DNA polymerase.

1 15. The method of claim 12, wherein the recognition site of the nicking
2 enzyme is at least 7 nucleotides in length and is present in one strand of the target sequence
3 about 9 times.

1 16. The method of claim 12, wherein the nicking enzyme is a type IIS
2 nicking enzyme.

1 17. The method of claim 12, wherein the nicking enzyme is a modified
2 type IIS nicking enzyme.

1 18. The method of claim 16, wherein the nicking enzyme is N.BbvC I.

1 19. The method of claim 12, further comprising:
2 (f) contacting the first extension primer to the second extension product under
3 conditions sufficient to allow the first extension primer to hybridize to the second extension
4 product and extending the first extension primer with the polymerase to generate a double
5 stranded product comprising restriction sites recognized by the nicking enzyme;
6 (g) contacting the double stranded product with the nicking enzyme under
7 conditions sufficient to allow the nicking enzyme to cleave a single strand of the double
8 stranded product, thereby generating a nicked double stranded product with a nick site on
9 each strand;

10 (h) contacting the first and second extension primer with the nicked double
11 stranded product under conditions sufficient to allow the first and second extension primers
12 to hybridize to the nicked double stranded product; and
13 (i) extending the first and second extension primers with a polymerase,
14 thereby releasing single stranded amplified target sequences into solution.

1 20. The method of claim 19, wherein the polymerase is a DNA polymerase
2 without 5'→3' exonuclease activity.

1 21. The method of claim 19, wherein the recognition site of the nicking
2 enzyme is at least 7 nucleotides in length and is present in one strand of the target sequence
3 about 9 times.

1 22. The method of claim 19, wherein the nicking enzyme is a type IIS
2 nicking enzyme.

1 23. The method of claim 19, wherein the nicking enzyme is Nt.BbvCI.

1 24. The method of claim 19, further comprising:
2 (h) detecting the amplified target sequence.

FIG. 1A

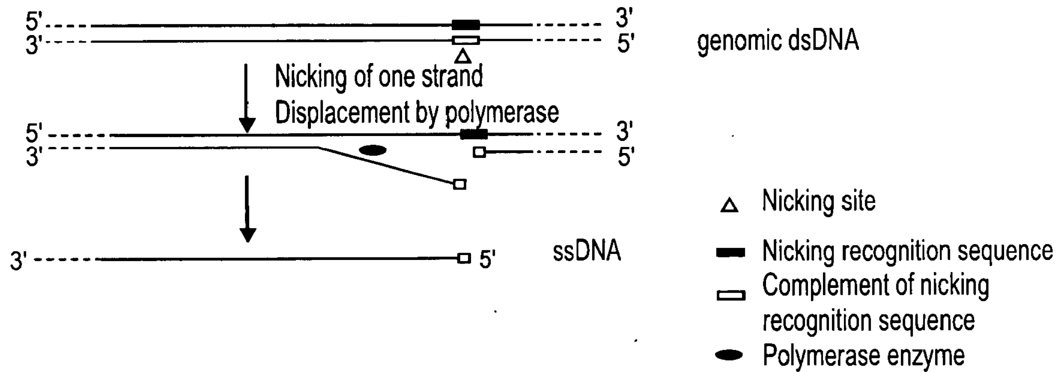
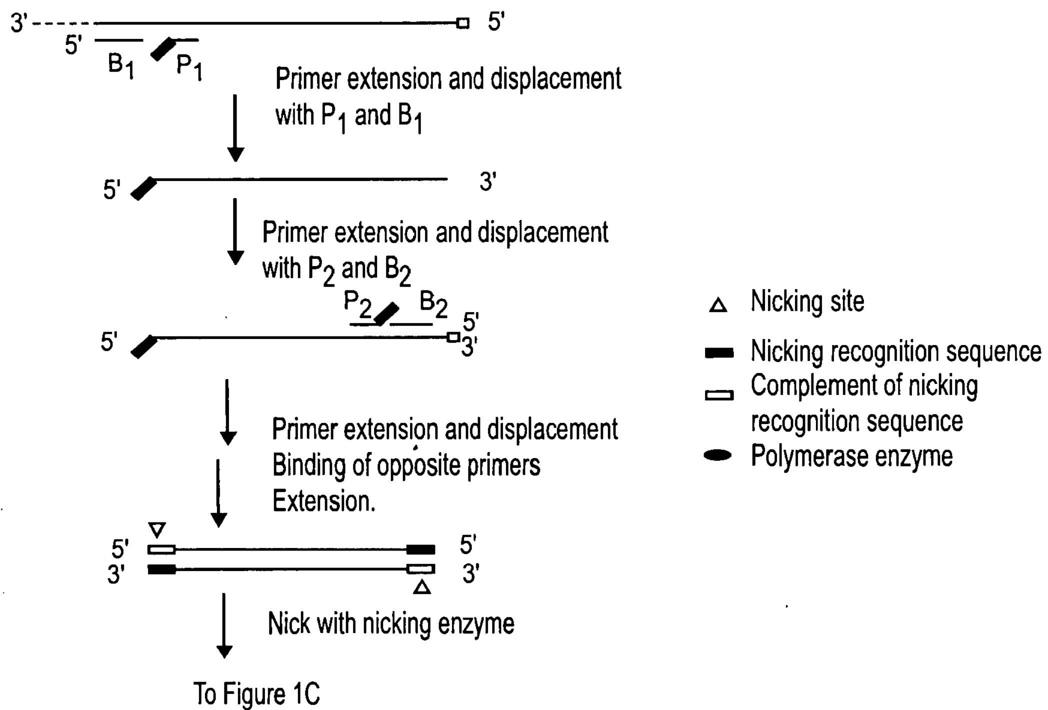
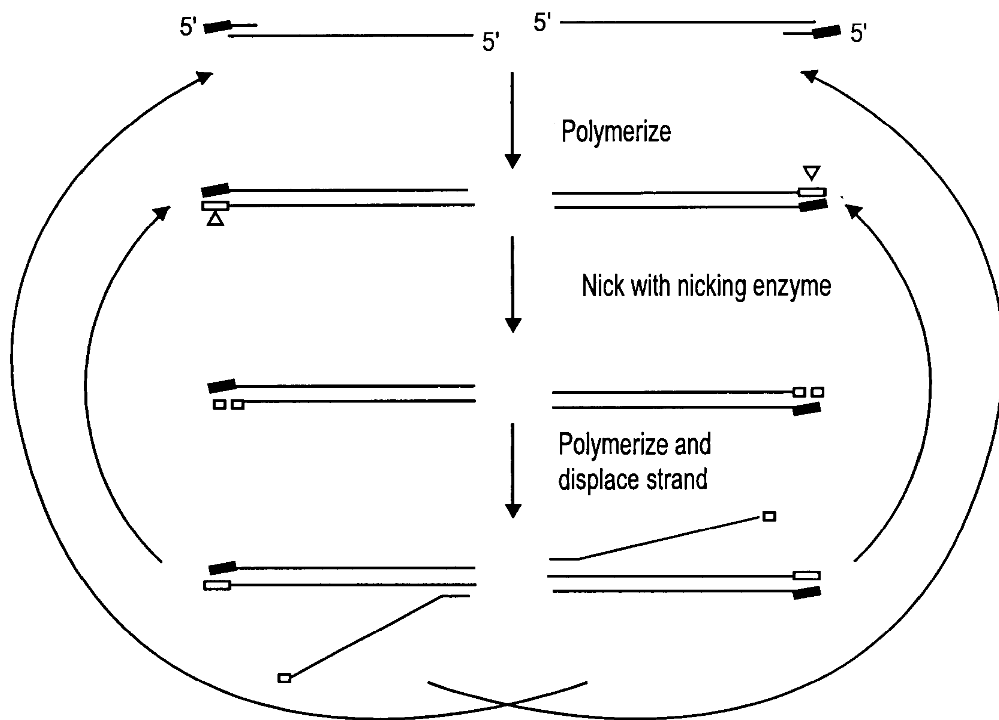


FIG. 1B



C. SDA Exponential Cycles



Hybridize SDA primers to the displaced strands

FIG. 1C

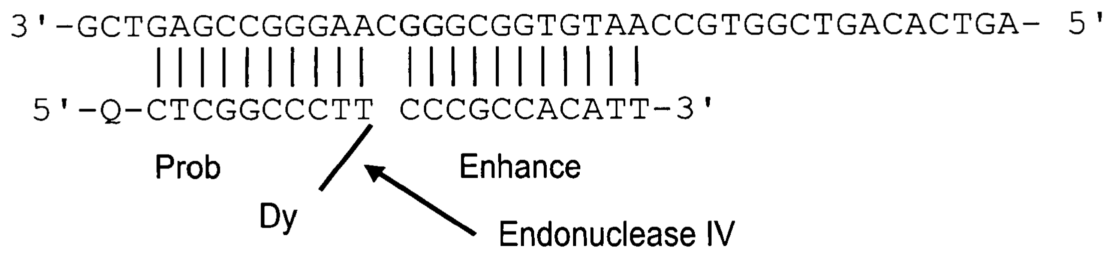


FIG. 2

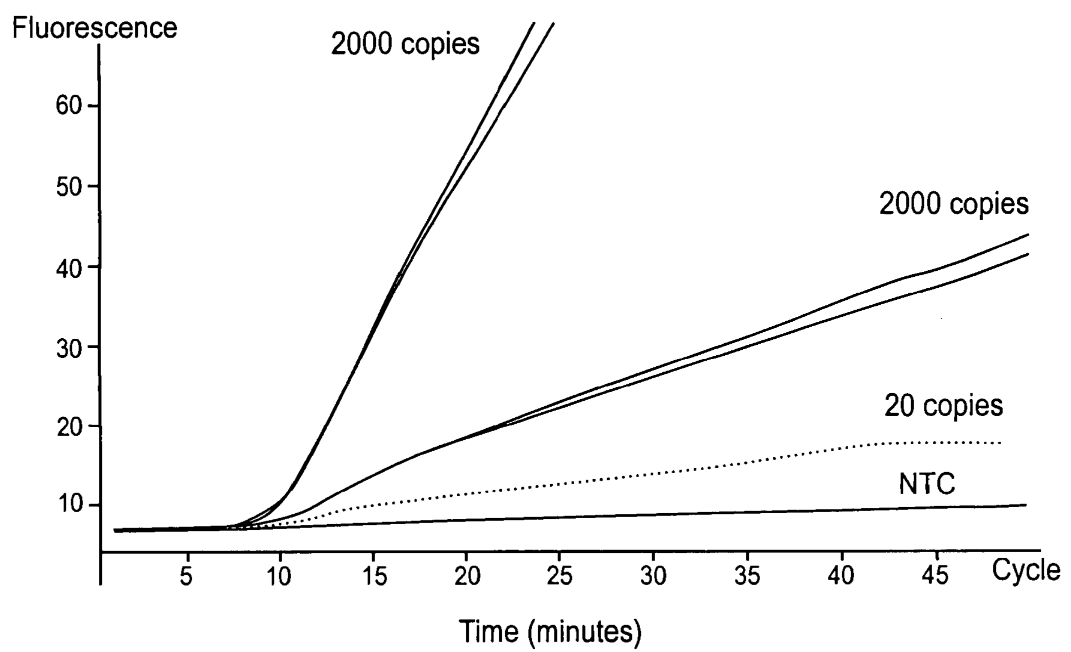
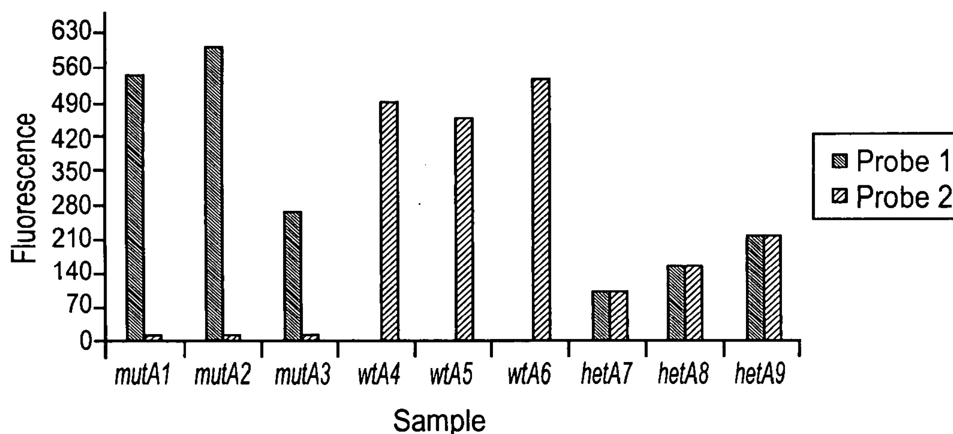


FIG. 3



Results for FVF2sda (FV Auto Low)					
Sample	**Red**	**Green**	**Pads**	**Ratio (R::G)**	**Probe Designation**
mutA1	550	11.95	1	46.02 : : 1	mut/mut
mutA2	609	13.54	1	44.96 : : 1	mut/mut
mutA3	271	7.17	1	37.79 : : 1	mut/mut
wtA4	1	491.59	1	1 : : 491.59	wt/wt
wtA5	0	458.13	1	Inf	wt/wt
wtA6	0	537.8	1	Inf	wt/wt
hetA7	98	98	1	1 : : 1	mut/wt
hetA8	155	156.96	1	1 : : 1.01	mut/wt
hetA9	223	223.89	1	1 : : 1	mut/wt

Probe Designation Thresholds:

Heterozygote < 1:2 < No Designation < 1:5 < Homozygote
Signal-to-noise ratio for *Pad Exclusion* or *No Designation:* 5:1
Minimum background-subtracted signal for *Pad Inclusion:* 50

Post-amplification genotype analysis of isothermal 1-step SDA product

FIG. 4

6/6

FIG. 5A

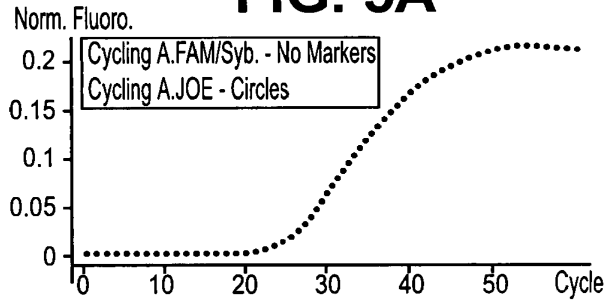


FIG. 5B

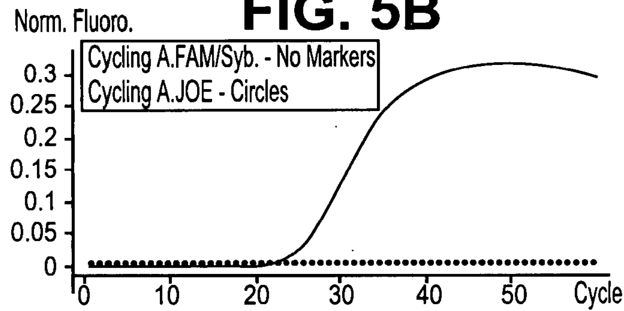


FIG. 5C

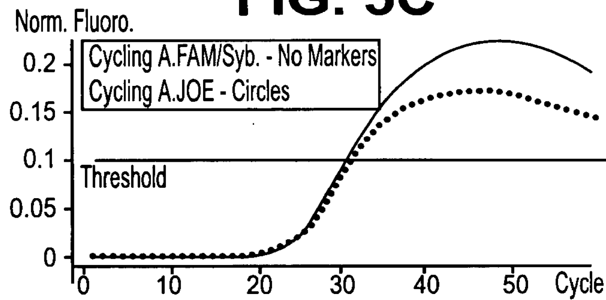
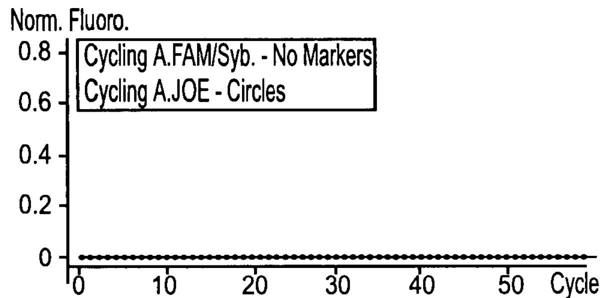


FIG. 5D



Real time genotype analysis of isothermal 1-step SDA products from human genomic DNA (A&B: homozygous wild type or mutant sample, C: heterozygous sample and D: no template control)

FIG. 5

(12) INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(19) World Intellectual Property Organization
International Bureau

(43) International Publication Date
3 January 2008 (03.01.2008)

PCT

(10) International Publication Number
WO 2008/002920 A3

(51) International Patent Classification:
C12Q 1/68 (2006.01) ***C07H 21/02*** (2006.01)
C12P 19/34 (2006.01) ***C07H 21/04*** (2006.01)

(21) International Application Number: PCT/US2007/072136

(22) International Filing Date: 26 June 2007 (26.06.2007)

(25) Filing Language: English

(26) Publication Language: English

(30) Priority Data:
60/805,847 26 June 2006 (26.06.2006) US

(71) Applicant *(for all designated States except US)*: **EPOCH BIOSCIENCES, INC.** [US/US]; 21720-23rd Dr., SE #150, Bothell, Washington 98021 (US).

(72) Inventors; and
(75) Inventors/Applicants *(for US only)*: **YAO, Zuxu** [CA/US]; 13330 Via Tresca #5, San Diego, California 92129 (US). **LIDGARD, Graham** [GB/US]; 432 Torrey Pines Rd., La Jolla, California 92037 (US). **BELOUSOV, Yevgeniy** [US/US]; 2717 144th Court SE, Mill Creek, Washington 98021 (US).

(74) Agents: FANG, Carol A. et al.; Townsend and Townsend and Crew, LLP., Two Embarcadero Center, Eighth Floor, San Francisco, California 94111-3834 (US).

(81) Designated States *(unless otherwise indicated, for every kind of national protection available)*: AE, AG, AL, AM, AT, AU, AZ, BA, BB, BG, BH, BR, BW, BY, BZ, CA, CH, CN, CO, CR, CU, CZ, DE, DK, DM, DO, DZ, EC, EE, EG, ES, FI, GB, GD, GE, GH, GM, GT, HN, HR, HU, ID, IL, IN, IS, JP, KE, KG, KM, KN, KP, KR, KZ, LA, LC, LK, LR, LS, LT, LU, LY, MA, MD, ME, MG, MK, MN, MW, MX, MY, MZ, NA, NG, NI, NO, NZ, OM, PG, PH, PL, PT, RO, RS, RU, SC, SD, SE, SG, SK, SL, SM, SV, SY, TJ, TM, TN, TR, TT, TZ, UA, UG, US, UZ, VC, VN, ZA, ZM, ZW.

(84) Designated States *(unless otherwise indicated, for every kind of regional protection available)*: ARIPO (BW, GH, GM, KE, LS, MW, MZ, NA, SD, SL, SZ, TZ, UG, ZM, ZW), Eurasian (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European (AT, BE, BG, CH, CY, CZ, DE, DK, EE, ES, FI, FR, GB, GR, HU, IE, IS, IT, LT, LU, LV, MC, MT, NL, PL, PT, RO, SE, SI, SK, TR), OAPI (BF, BJ, CF, CG, CI, CM, GA, GN, GQ, GW, ML, MR, NE, SN, TD, TG).

Published:
— *with international search report*
— *before the expiration of the time limit for amending the claims and to be republished in the event of receipt of amendments*

(88) Date of publication of the international search report: 20 November 2008

WO 2008/002920 A3

(54) Title: METHODS FOR GENERATING TARGET NUCLEIC ACID SEQUENCES

(57) Abstract: The present invention provides methods of generating target nucleic acids for amplification using nicking enzymes and methods for amplifying the generated target nucleic acids.

INTERNATIONAL SEARCH REPORT

International application No.

PCT/US07/72136

A. CLASSIFICATION OF SUBJECT MATTER

IPC: **C12Q 1/68**(2006.01);**C12P 19/34**(2006.01)
C07H 21/02(2006.01),21/04(2006.01)

USPC: 435/6,91.2;536/23.1,24.3

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
U.S. : 435/6, 91.2; 536/23.1, 24.3

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)
WEST, PubMed

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X --- Y	US 2003/0104431 A1 (VAN NESS et al.) 5 June 2003; page 1, [0008]-[0011]; page 2, [0018]; page 3, [0026]; page 6, [0059]-[0061]; page 7, [0062]-[0065];	1-11 ----------- 12-24
Y	US 6,191,267 B1 (KONG et al.) 20 February 2001; col. 2, lines 43-48; col. 14, lines 10-67; col. 15, 16	12-24

☐ Further documents are listed in the continuation of Box C. ☐ See patent family annex.

* Special categories of cited documents:

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier application or patent published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art

"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
25 August 2008 (25.08.2008)	16 SEP 2008
Name and mailing address of the ISA/US Mail Stop PCT, Attn: ISA/US Commissioner for Patents P.O. Box 1450 Alexandria, Virginia 22313-1450 Facsimile No. (571) 273-3201	Authorized officer TERESA E. STRZELECKA Telephone No. (703) 308-0196

Form PCT/ISA/210 (second sheet) (April 2007)

Electronic Patent Application Fee Transmittal

Application Number:	14067620
Filing Date:	30-Oct-2013
Title of Invention:	Nicking and Extension Amplification Reaction for the Exponential Amplification of Nucleic Acids
First Named Inventor/Applicant Name:	Brian K. Maples
Filer:	Ian J.S. Lodovice/Mary Florczak
Attorney Docket Number:	30171-0025002 / ITI-001

Filed as Large Entity

Filing Fees for **Utility under 35 USC 111(a)**

Description	Fee Code	Quantity	Amount	Sub-Total in USD($)
Basic Filing:				
PETITION FEE- 37 CFR 1.17(H) (GROUP III)	1464	1	140	140
RCE- 2ND AND SUBSEQUENT REQUEST	1820	1	1700	1700
Pages:				
Claims:				
Miscellaneous-Filing:				
Petition:				
Patent-Appeals-and-Interference:				

Description	Fee Code	Quantity	Amount	Sub-Total in USD($)
Post-Allowance-and-Post-Issuance:				
Extension-of-Time:				
Miscellaneous:				
		Total in USD ($)		**1840**

UNITED STATES PATENT AND TRADEMARK OFFICE

Commissioner for Patents
United States Patent and Trademark Office
P.O. Box 1450
Alexandria, VA 22313-1450
www.uspto.gov

Decision Date : November 1, 2016

In re Application of :

Brian Maples

DECISION ON PETITION
UNDER CFR 1.313(c)(2)

Application No : 14067620

Filed : 30-Oct-2013

Attorney Docket No : 30171-0025002 / ITI-001

This is an electronic decision on the petition under 37 CFR 1.313(c)(2), filed November 1, 2016, to withdraw the above-identified application from issue after payment of the issue fee.

The petition is **GRANTED.**

The above-identified application is withdrawn from issue for consideration of a submission under 37 CFR 1.114 (request for continued examination). See 37 CFR 1.313(c)(2).

Petitioner is advised that the issue fee paid in this application cannot be refunded. If, however, this application is again allowed, petitioner may request that it be applied towards the issue fee required by the new Notice of Allowance.

Telephone inquiries concerning this decision should be directed to the Patent Electronic Business Center (EBC) at 866-217-9197.

This application file is being referred to Technology Center AU 1637 for processing of the request for continuing examination under 37 CFR 1.114 .

Office of Petitions

Electronic Acknowledgement Receipt

EFS ID:	27388770
Application Number:	14067620
International Application Number:	
Confirmation Number:	4288
Title of Invention:	Nicking and Extension Amplification Reaction for the Exponential Amplification of Nucleic Acids
First Named Inventor/Applicant Name:	Brian K. Maples
Customer Number:	26161
Filer:	Ian J.S. Lodovice/Mary Florczak
Filer Authorized By:	Ian J.S. Lodovice
Attorney Docket Number:	30171-0025002 / ITI-001
Receipt Date:	01-NOV-2016
Filing Date:	30-OCT-2013
Time Stamp:	17:20:41
Application Type:	Utility under 35 USC 111(a)

Payment information:

Submitted with Payment	yes
Payment Type	DA
Payment was successfully received in RAM	$1840
RAM confirmation Number	110216INTEFSW00003983061050
Deposit Account	
Authorized User	

The Director of the USPTO is hereby authorized to charge indicated fees and credit any overpayment as follows:

File Listing:

Document Number	Document Description	File Name	File Size(Bytes)/ Message Digest	Multi Part /.zip	Pages (if appl.)
1	Petition automatically granted by EFS	petition-request.pdf	31416 f867ec07ed3143522ca79474cd03d806887 08a60	no	2
Warnings:					
Information:					
2	Request for Continued Examination (RCE)	301710025002RCE.pdf	161267 0e3a81d370443338a2ebd11e845821ad24 1070ee	no	1
Warnings:					
This is not a USPTO supplied RCE SB30 form.					
Information:					
3	Quick Path Information Disclosure Statement	301710025002IDS.pdf	202745 98e3faf3d2dd7fab9cad1771add8cbf6db02 11a7	no	2
Warnings:					
Information:					
4	Foreign Reference	WO2008002920.pdf	2507081 924ebb9831d575f174fc08806f8c835fd54f6 316	no	49
Warnings:					
Information:					
5	Non Patent Literature	30171OA1.pdf	244974 270faab1abe570ebd7b3f6f5639d423089ff aeb8	no	31
Warnings:					
Information:					
6	Non Patent Literature	30171Response.pdf	341659 4743edaf1f04516711c2bb51d8e726e40ee 4a1a1	no	21
Warnings:					

The PDF file has been signed with a digital signature and the legal effect of the document will be based on the contents of the file not the digital signature.

Information:

7	Non Patent Literature	30171OA.pdf	281453 1382418baeb4e0824d7e855001af0ac04ff4ed54	no	29

Warnings:

Information:

8	Non Patent Literature	McDowell.pdf	976483 983c9da154271928278077350a608996ec27079d	no	8

Warnings:

Information:

9	Non Patent Literature	AUOA.pdf	149471 0b89de69f8b7f72491ea5d12de1f825994ce8b9a	no	4

Warnings:

Information:

10	Non Patent Literature	30171Buck.pdf	793535 0dbb1a15b6060188a0525b91e9d2b652cbbd9891	no	9

Warnings:

Information:

11	Non Patent Literature	30171Hite.pdf	825456 c7db43484297aec10761b993765a596ce0aac312	no	6

Warnings:

Information:

12	Fee Worksheet (SB06)	fee-info.pdf	32658 d5f76edb36f79c43304aadc6767f431493c779da	no	2

Warnings:

Information:

Total Files Size (in bytes):	6548198

This Acknowledgement Receipt evidences receipt on the noted date by the USPTO of the indicated documents, characterized by the applicant, and including page counts, where applicable. It serves as evidence of receipt similar to a Post Card, as described in MPEP 503.

New Applications Under 35 U.S.C. 111
If a new application is being filed and the application includes the necessary components for a filing date (see 37 CFR 1.53(b)-(d) and MPEP 506), a Filing Receipt (37 CFR 1.54) will be issued in due course and the date shown on this Acknowledgement Receipt will establish the filing date of the application.

National Stage of an International Application under 35 U.S.C. 371
If a timely submission to enter the national stage of an international application is compliant with the conditions of 35 U.S.C. 371 and other applicable requirements a Form PCT/DO/EO/903 indicating acceptance of the application as a national stage submission under 35 U.S.C. 371 will be issued in addition to the Filing Receipt, in due course.

New International Application Filed with the USPTO as a Receiving Office
If a new international application is being filed and the international application includes the necessary components for an international filing date (see PCT Article 11 and MPEP 1810), a Notification of the International Application Number and of the International Filing Date (Form PCT/RO/105) will be issued in due course, subject to prescriptions concerning national security, and the date shown on this Acknowledgement Receipt will establish the international filing date of the application.

Doc Code: PET.AUTO Document Description: Petition automatically granted by EFS-Web	PTO/SB/140 U.S. Patent and Trademark Office Department of Commerce

Electronic Petition Request	**PETITION TO WITHDRAW AN APPLICATION FROM ISSUE AFTER PAYMENT OF THE ISSUE FEE UNDER 37 CFR 1.313(c)**
Application Number	14067620
Filing Date	30-Oct-2013
First Named Inventor	Brian Maples
Art Unit	1637
Examiner Name	ANGELA BERTAGNA
Attorney Docket Number	30171-0025002 / ITI-001
Title	Nicking and Extension Amplification Reaction for the Exponential Amplification of Nucleic Acids

An application may be withdrawn from issue for further action upon petition by the applicant. To request that the Office withdraw an application from issue, applicant must file a petition under this section including the fee set forth in § 1.17(h) and a showing of good and sufficient reasons why withdrawal of the application from issue is necessary.

APPLICANT HEREBY PETITIONS TO WITHDRAW THIS APPLICATION FROM ISSUE UNDER 37 CFR 1.313(c).

A grantable petition requires the following items:
(1) Petition fee; and
(2) One of the following reasons:
(a) Unpatentability of one or more claims, which must be accompanied by an unequivocal statement that one or more claims are unpatentable, an amendment to such claim or claims, and an explanation as to how the amendment causes such claim or claims to be patentable;
(b) Consideration of a request for continued examination in compliance with § 1.114 (for a utility or plant application only); or
(c) Express abandonment of the application. Such express abandonment may be in favor of a continuing application, but not a CPA under 37 CFR 1.53(d).

Petition Fee

- ○ Small Entity
- ○ Micro Entity
- ◉ Regular Undiscounted

Reason for withdrawal from issue

○ One or more claims are unpatentable

◉ Consideration of a request for continued examination (RCE) (List of Required Documents and Fees)

○ Applicant hereby expressly abandons the instant application (any attorney/agent signing for this reason must have power of attorney pursuant to 37 CFR 1.32(b)).

RCE request,submission, and fee.

☐ I certify, in accordance with 37 CFR 1.4(d)(4) that :
The RCE request ,submission, and fee have already been filed in the above-identified application on

☒ Are attached.

THIS PORTION MUST BE COMPLETED BY THE SIGNATORY OR SIGNATORIES

I certify, in accordance with 37 CFR 1.4(d)(4) that I am:

◉ An attorney or agent registered to practice before the Patent and Trademark Office who has been given power of attorney in this application.

○ An attorney or agent registered to practice before the Patent and Trademark Office, acting in a representative capacity.

○ A sole inventor

○ A joint inventor; I certify that I am authorized to sign this submission on behalf of all of the inventors as evidenced by the power of attorney in the application

○ A joint inventor; all of whom are signing this e-petition

Signature	/Ian J.S. Lodovice, Reg. No. 59,749/
Name	Ian J. Lodovice
Registration Number	59749

Document code: WFEE

United States Patent and Trademark Office
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01 FC : 1806 180.00 DA

Document code: WFEE

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Seq No: 1 Sales Acctg Dt: 11/08/2016 061050 14067620
01 FC : 1806 180.00 CR

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APPLICATION NO.	ISSUE DATE	PATENT NO.	ATTORNEY DOCKET NO.	CONFIRMATION NO.
14/067,620	11/08/2016	9487826	30171-0025002 / ITI-001	4288

26161 7590 10/19/2016

FISH & RICHARDSON P.C. (BO)
P.O. BOX 1022
MINNEAPOLIS, MN 55440-1022

ISSUE NOTIFICATION

The projected patent number and issue date are specified above.

Determination of Patent Term Adjustment under 35 U.S.C. 154 (b)
(application filed on or after May 29, 2000)

The Patent Term Adjustment is 0 day(s). Any patent to issue from the above-identified application will include an indication of the adjustment on the front page.

If a Continued Prosecution Application (CPA) was filed in the above-identified application, the filing date that determines Patent Term Adjustment is the filing date of the most recent CPA.

Applicant will be able to obtain more detailed information by accessing the Patent Application Information Retrieval (PAIR) WEB site (http://pair.uspto.gov).

Any questions regarding the Patent Term Extension or Adjustment determination should be directed to the Office of Patent Legal Administration at (571)-272-7702. Questions relating to issue and publication fee payments should be directed to the Application Assistance Unit (AAU) of the Office of Data Management (ODM) at (571)-272-4200.

APPLICANT(s) (Please see PAIR WEB site http://pair.uspto.gov for additional applicants):

Ionian Technologies, Inc., San Diego, CA;
Brian K. Maples, Lake Forest, CA;
Rebecca C. Holmberg, San Diego, CA;
Andrew P. Miller, San Diego, CA;
Jarrod Provins, Dana Point, CA;
Richard Roth, Carlsbad, CA;
Jeffrey Mandell, San Diego, CA;

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IR103 (Rev. 10/09)

PTO/SB/08A(08-03)
Approved for use through 07/31/2006. OMB 0651-0031
US Patent & Trademark Office: U.S. DEPARTMENT OF COMMERCE
Under the Paperwork Reduction Act of 1995, no persons are required to respond to a collection of information unless it contains a valid OMB control number.

Substitute for form 1449A/PTO	*Complete if Known*	
INFORMATION DISCLOSURE STATEMENT BY APPLICANT *(Use as many sheets as necessary)*	**Application Number**	11/778,018
	Filing Date	July 14, 2007
	First Named Inventor	MAPLES, Brian K.
	Group Art Unit	1635
	Examiner Name	NOT YET ASSIGNED
Sheet 1 of 2	Attorney Docket No: ITI-1001-UT	

Change(s) applied to document, /A.B./
/P.A.P./ 9/30/2016
Change(s) applied to document, /S.X.R./ 10/4/2016

US PATENT DOCUMENTS

Examiner Initials *	Cite No.[1]	USP Document Number	Publication Date	Name of Patentee or Applicant of cited Document	Pages,Columns,Lines,Where Relevant Passages or Relevant Figures Appear
/A.B./ (arrow through A17)	**A1.**	5,681,705	10/28/1997	~~Becton, Dickinson and Company~~	Schram, et al.
	A2.	5,928,869	7/27/1999	~~Becton, Dickinson and Company~~	Nadeau, et al.
	A3.	6,294,337	9/25/2001	~~Riken~~ Hayashizaki	
	A4.	6,372,434	4/16/2002	~~Molecular Staging, Inc.~~	Weissman, et al.
	A5.	RE39885	10/16/2007	~~Becton, Dickinson and Company~~	Nadeau, et al.
	A6.	US2002/0042059	4/11/2002	~~The Regents of the University of Michigan~~	Makarov, et al.
	A7.	US2002/0150919	10/17/2002	~~K-TEC INC.~~	Weismann, et al.
	A8.	US2003/0165911	9/4/2003	~~Keck Graduate Institute~~	Schmitz, et al.
	A9.	US2005/0009050	1/13/2005	~~GENZYME CORP~~	Nadeau, et al.
	A10.	US2005/0042601	2/24/2005	~~IMMUNEREGEN BIOSCIENCES INC~~	Wolfe
	A11.	US2005/0112639	5/26/2005	WANG et al.	
	A12.	US2005/0147973	7/7/2005	~~AXCELIS TECH INC~~	Knott
	A13.	US2005/0164207	7/28/2005	~~Affymetrix, INC.~~	Shapero
	A14.	US2005/0202490	9/15/2005	MAKAROV et al.	
	A15.	US2005/0233332	10/20/2005	COLLIS	
	A16.	US2005/0266417	12/1/2005	BARANY et al.	
	A17.	US2007/0020639	1/25/2007	~~Affymetrix, INC.~~	Shapero
/A.B./	**A18.**	US2007/0031857	2/8/2007	~~Rubicon Genomics, Inc.~~	Makarov, et al.

EXAMINER /Angela Bertagna/ **DATE CONSIDERED** 10/10/2014

Substitute Disclosure Statement Form (PTO-1449)
* EXAMINER: Initial if reference considered, whether or not citation is in conformance with MPEP 609. Draw line through citation if not in conformance and not considered. Include copy of this form with next communication to applicant. 1 Applicant's unique citation designation number (optional) 2 Applicant is to place a check mark here if English language Translation is attached

Substitute Disclosure Form / U.S. Department of Commerce Patent and Trademark Office	Attorney Docket No. 30171-0025002	Application No. 14/067,620
Information Disclosure Statement by Applicant (Use several sheets if necessary)	First Named Inventor Brian K. Maples	
(37 CFR §1.98(b))	Filing Date October 30, 2013	Group Art Unit 1637

U.S. Patent Documents

Examiner Initial	Desig. ID	Document Number	Publication Date	Patentee	Class	Subclass	Filing Date If Appropriate
	1						

Foreign Patent Documents or Published Foreign Patent Applications

Examiner Initial	Desig. ID	Document Number	Publication Date	Country or Patent Office	Class	Subclass	Translation Yes	Translation No
/A.B./	2	EP2660333	11/06/2013	Europe				
/A.B./	3	EP2657350	10/30/2013	Europe				
/A.B./	4	WO2007/028833	03/15/2007	WIPO				
/A.B./	5	WO94/03635	~~02/17/1997~~ 02/1994	WIPO				
	6							

Change(s) applied to document, /S.X.R./ 10/5/2016

Other Documents (include Author, Title, Date, and Place of Publication)

Examiner Initial	Desig. ID	Document
/A.B./	7	Sequence of vector pUC19, downloaded from http://genome-www.stanford.edu/vectordb/vector_descrip/COMPLET... On March 27, 2014
/A.B./	8	Notice of Opposition in corresponding EP Application No. 08781827.4, dated May 6, 2014, pages 1-36
	9	

Examiner Signature /Angela Bertagna/	Date Considered 10/10/2014
EXAMINER: Initials citation considered. Draw line through citation if not in conformance and not considered. Include copy of this form with next communication to applicant.	

Substitute Disclosure Form

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

First Named Inventor	:	Brian K. Maples	Art Unit	:	1637
Serial No.	:	14/067,620	Examiner	:	Angela Marie Bertagna
Filed	:	October 30, 2013	Confirmation No.	:	4288
			Notice of Allowance Date: September 16, 2016		
Title	:	NICKING AND EXTENSION AMPLIFICATION REACTION FOR THE EXPONENTIAL AMPLIFICATION OF NUCLEIC ACIDS			

MAIL STOP ISSUE FEE
Commissioner for Patents
P.O. Box 1450
Alexandria, VA 22313-1450

REPLY TO NOTICE OF ALLOWANCE

In response to the Notice of Allowance dated September 16, 2016, enclosed is a completed Part B - Fee(s) Transmittal.

The issue fee is being paid with this reply on the Electronic Filing System. Apply those fees and any other necessary charges or credits to Deposit Account 06 1050, referencing the above attorney docket number.

Respectfully submitted,

Date: September 21, 2016

/Ian J.S. Lodovice, Reg. No. 59,749/
Ian J. Lodovice
Reg. No. 59,749

Customer Number 26161
Fish & Richardson P.C.
Telephone: (617) 956-5972
Facsimile: (877) 769-7945

23567492.doc

PART B - FEE(S) TRANSMITTAL

Complete and send this form, together with applicable fee(s), to: Mail Mail Stop ISSUE FEE
Commissioner for Patents
P.O. Box 1450
Alexandria, Virginia 22313-1450
or Fax (571)-273-2885

INSTRUCTIONS: This form should be used for transmitting the ISSUE FEE and PUBLICATION FEE (if required). Blocks 1 through 5 should be completed where appropriate. All further correspondence including the Patent, advance orders and notification of maintenance fees will be mailed to the current correspondence address as indicated unless corrected below or directed otherwise in Block 1, by (a) specifying a new correspondence address; and/or (b) indicating a separate "FEE ADDRESS" for maintenance fee notifications.

CURRENT CORRESPONDENCE ADDRESS (Note: Use Block 1 for any change of address)

26161 7590 09/16/2016

FISH & RICHARDSON P.C.
P.O. BOX 1022
MINNEAPOLIS, MN 55440-1022

Note: A certificate of mailing can only be used for domestic mailings of the Fee(s) Transmittal. This certificate cannot be used for any other accompanying papers. Each additional paper, such as an assignment or formal drawing, must have its own certificate of mailing or transmission.

Certificate of Mailing or Transmission

I hereby certify that this Fee(s) Transmittal is being deposited with the United States Postal Service with sufficient postage for first class mail in an envelope addressed to the Mail Stop ISSUE FEE address above, or being facsimile transmitted to the USPTO (571) 273-2885, on the date indicated below.

(Depositor's name)
(Signature)
(Date)

APPLICATION NO.	FILING DATE	FIRST NAMED INVENTOR	ATTORNEY DOCKET NO.	CONFIRMATION NO.
14/067,620	10/30/2013	Brian K. Maples	30171-0025002	4288

TITLE OF INVENTION: NICKING AND EXTENSION AMPLIFICATION REACTION FOR THE EXPONENTIAL AMPLIFICATION OF NUCLEIC ACIDS

APPLN. TYPE	ENTITY STATUS	ISSUE FEE DUE	PUBLICATION FEE DUE	PREV. PAID ISSUE FEE	TOTAL FEE(S) DUE	DATE DUE
nonprovisional	LARGE	$960	$0		$960	12/16/2016

EXAMINER	ART UNIT	CLASS-SUBCLASS
BERTAGNA, ANGELA MARIE	1637	435-006120

1. Change of correspondence address or indication of "Fee Address" (37 CFR 1.363).

[ ] Change of correspondence address (or Change of Correspondence Address form PTO/SB/122) attached.

[ ] "Fee Address" indication (or "Fee Address" Indication form PTO/SB/47; Rev 03-02 or more recent) attached. **Use of a Customer Number is required.**

2. For printing on the patent front page, list

(1) the names of up to 3 registered patent attorneys or agents OR, alternatively,

(2) the name of a single firm (having as a member a registered attorney or agent) and the names of up to 2 registered patent attorneys or agents. If no name is listed, no name will be printed.

1 Fish & Richardson P.C.
2 ______
3 ______

3. ASSIGNEE NAME AND RESIDENCE DATA TO BE PRINTED ON THE PATENT (print or type)

PLEASE NOTE: Unless an assignee is identified below, no assignee data will appear on the patent. If an assignee is identified below, the document has been filed for recordation as set forth in 37 CFR 3.11. Completion of this form is NOT a substitute for filing an assignment.

(A) NAME OF ASSIGNEE
Ionian Technologies, Inc.

(B) RESIDENCE: (CITY and STATE OR COUNTRY)
San Diego, CA

Please check the appropriate assignee category or categories (will not be printed on the patent): [ ] Individual [X] Corporation or other private group entity [ ] Government

4a. The following fee(s) are submitted:

[X] Issue Fee
[ ] Publication Fee (No small entity discount permitted)
[ ] Advance Order - # of Copies ______

4b. Payment of Fee(s): **(Please first reapply any previously paid issue fee shown above)**

[ ] A check in the amount of the fee(s) is enclosed.
[ ] Payment by credit card. Form PTO-2038 is attached.
[X] The Director is hereby authorized to charge the required fee(s), or credit any overpayment, to Deposit Account Number 06-1050

5. **Change in Entity Status** (from status indicated above)

[ ] Applicant certifying micro entity status. See 37 CFR 1.29

NOTE: Absent a valid certification of Micro Entity Status (see form PTO/SB/15A and 15B), issue fee payment in the micro entity amount will not be accepted at the risk of application abandonment.

[ ] Applicant asserting small entity status. See 37 CFR 1.27.

NOTE: If the application was previously under micro entity status, checking this box will be taken to be a notification of loss of entitlement to micro entity status.

[ ] Applicant changing to regular undiscounted fee status.

NOTE: Checking this box will be taken to be a notification of loss of entitlement to small or micro entity status, as applicable.

The Director of the USPTO is requested to apply the Issue Fee and Publication Fee (if any) or to re-apply any previously paid issue fee to the application identified above.
NOTE: The Issue Fee and Publication Fee (if required) will not be accepted from anyone other than the applicant; a registered attorney or agent; or the assignee or other party in interest as shown by the records of the United States Patent and Trademark Office.

Authorized Signature /Ian J.S. Lodovice, Reg. No. 59,749/ Date September 21, 2016

Typed or printed name Ian J. Lodovice Registration No. 59,749

23568536.doc

Electronic Patent Application Fee Transmittal

Application Number:	14067620
Filing Date:	30-Oct-2013
Title of Invention:	Nicking and Extension Amplification Reaction for the Exponential Amplification of Nucleic Acids
First Named Inventor/Applicant Name:	Brian K. Maples
Filer:	Ian J.S. Lodovice/Mary Florczak
Attorney Docket Number:	30171-0025002 / ITI-001

Filed as Large Entity

Filing Fees for **Utility under 35 USC 111(a)**

Description	Fee Code	Quantity	Amount	Sub-Total in USD($)
Basic Filing:				
Pages:				
Claims:				
Miscellaneous-Filing:				
Petition:				
Patent-Appeals-and-Interference:				
Post-Allowance-and-Post-Issuance:				

Description	Fee Code	Quantity	Amount	Sub-Total in USD($)
UTILITY APPL ISSUE FEE	1501	1	960	960
PUBL. FEE- EARLY, VOLUNTARY, OR NORMAL	1504	1	0	0
Extension-of-Time:				
Miscellaneous:				
		Total in USD ($)		**960**

Electronic Acknowledgement Receipt

EFS ID:	26996800
Application Number:	14067620
International Application Number:	
Confirmation Number:	4288
Title of Invention:	Nicking and Extension Amplification Reaction for the Exponential Amplification of Nucleic Acids
First Named Inventor/Applicant Name:	Brian K. Maples
Customer Number:	26161
Filer:	Ian J.S. Lodovice/Denise Siede
Filer Authorized By:	Ian J.S. Lodovice
Attorney Docket Number:	30171-0025002 / ITI-001
Receipt Date:	21-SEP-2016
Filing Date:	30-OCT-2013
Time Stamp:	18:05:58
Application Type:	Utility under 35 USC 111(a)

Payment information:

Submitted with Payment	yes
Payment Type	DA
Payment was successfully received in RAM	$960
RAM confirmation Number	092216INTEFSW00005299061050
Deposit Account	
Authorized User	

The Director of the USPTO is hereby authorized to charge indicated fees and credit any overpayment as follows:

File Listing:

Document Number	Document Description	File Name	File Size(Bytes)/ Message Digest	Multi Part /.zip	Pages (if appl.)
1	Applicant summary of interview with examiner	301710025002IS.pdf	71143 627e5b8c09735b5ce0b11914a71f762ad0d98a0a	no	1
Warnings:					
Information:					
2	Issue Fee Payment (PTO-85B)	301710025002IssueFee.pdf	196326 272ee52806359f2ca32b0db7df783c8d4d6e8513	no	2
Warnings:					
Information:					
3	Fee Worksheet (SB06)	fee-info.pdf	32377 7e50647332369456f21f6016ef28ffe9cfc1a6fd	no	2
Warnings:					
Information:					
			Total Files Size (in bytes):	299846	

This Acknowledgement Receipt evidences receipt on the noted date by the USPTO of the indicated documents, characterized by the applicant, and including page counts, where applicable. It serves as evidence of receipt similar to a Post Card, as described in MPEP 503.

New Applications Under 35 U.S.C. 111
If a new application is being filed and the application includes the necessary components for a filing date (see 37 CFR 1.53(b)-(d) and MPEP 506), a Filing Receipt (37 CFR 1.54) will be issued in due course and the date shown on this Acknowledgement Receipt will establish the filing date of the application.

National Stage of an International Application under 35 U.S.C. 371
If a timely submission to enter the national stage of an international application is compliant with the conditions of 35 U.S.C. 371 and other applicable requirements a Form PCT/DO/EO/903 indicating acceptance of the application as a national stage submission under 35 U.S.C. 371 will be issued in addition to the Filing Receipt, in due course.

New International Application Filed with the USPTO as a Receiving Office
If a new international application is being filed and the international application includes the necessary components for an international filing date (see PCT Article 11 and MPEP 1810), a Notification of the International Application Number and of the International Filing Date (Form PCT/RO/105) will be issued in due course, subject to prescriptions concerning national security, and the date shown on this Acknowledgement Receipt will establish the international filing date of the application.

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

First Named Inventor	:	Brian K. Maples	Art Unit	:	1637
Serial No.	:	14/067,620	Examiner	:	Angela Marie Bertagna
Filed	:	October 30, 2013	Conf. No.	:	4288
Title	:	NICKING AND EXTENSION AMPLIFICATION REACTION FOR THE EXPONENTIAL AMPLIFICATION OF NUCLEIC ACIDS			

Mail Stop Amendment
Commissioner for Patents
P.O. Box 1450
Alexandria, VA 22313-1450

INTERVIEW SUMMARY

The undersigned representative thanks the Examiner for the courtesy of the telephonic interview held on August 26, 2016.

The substance of the interview included a discussion of claims 67-83, 85-88, 90, 92 and 95-107.

Respectfully submitted,

Date: September 21, 2016________

/Ian J.S. Lodovice, Reg. No. 59,749/
Ian J. Lodovice
Reg. No. 59,749

Customer Number 26161
Fish & Richardson P.C.
Telephone: (617) 956-5972
Facsimile: (877) 769-7945

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UNITED STATES PATENT AND TRADEMARK OFFICE

UNITED STATES DEPARTMENT OF COMMERCE
United States Patent and Trademark Office
Address: COMMISSIONER FOR PATENTS
P.O. Box 1450
Alexandria, Virginia 22313-1450
www.uspto.gov

NOTICE OF ALLOWANCE AND FEE(S) DUE

26161 7590 09/16/2016
FISH & RICHARDSON P.C. (BO)
P.O. BOX 1022
MINNEAPOLIS, MN 55440-1022

EXAMINER	
BERTAGNA, ANGELA MARIE	
ART UNIT	PAPER NUMBER
1637	

DATE MAILED: 09/16/2016

APPLICATION NO.	FILING DATE	FIRST NAMED INVENTOR	ATTORNEY DOCKET NO.	CONFIRMATION NO.
14/067,620	10/30/2013	Brian K. Maples	30171-0025002 / ITI-001	4288

TITLE OF INVENTION: Nicking and Extension Amplification Reaction for the Exponential Amplification of Nucleic Acids

APPLN. TYPE	ENTITY STATUS	ISSUE FEE DUE	PUBLICATION FEE DUE	PREV. PAID ISSUE FEE	TOTAL FEE(S) DUE	DATE DUE
nonprovisional	UNDISCOUNTED	$960	$0	$0	$960	12/16/2016

THE APPLICATION IDENTIFIED ABOVE HAS BEEN EXAMINED AND IS ALLOWED FOR ISSUANCE AS A PATENT. PROSECUTION ON THE MERITS IS CLOSED. THIS NOTICE OF ALLOWANCE IS NOT A GRANT OF PATENT RIGHTS. THIS APPLICATION IS SUBJECT TO WITHDRAWAL FROM ISSUE AT THE INITIATIVE OF THE OFFICE OR UPON PETITION BY THE APPLICANT. SEE 37 CFR 1.313 AND MPEP 1308.

THE ISSUE FEE AND PUBLICATION FEE (IF REQUIRED) MUST BE PAID WITHIN THREE MONTHS FROM THE MAILING DATE OF THIS NOTICE OR THIS APPLICATION SHALL BE REGARDED AS ABANDONED. THIS STATUTORY PERIOD CANNOT BE EXTENDED. SEE 35 U.S.C. 151. THE ISSUE FEE DUE INDICATED ABOVE DOES NOT REFLECT A CREDIT FOR ANY PREVIOUSLY PAID ISSUE FEE IN THIS APPLICATION. IF AN ISSUE FEE HAS PREVIOUSLY BEEN PAID IN THIS APPLICATION (AS SHOWN ABOVE), THE RETURN OF PART B OF THIS FORM WILL BE CONSIDERED A REQUEST TO REAPPLY THE PREVIOUSLY PAID ISSUE FEE TOWARD THE ISSUE FEE NOW DUE.

HOW TO REPLY TO THIS NOTICE:

I. Review the ENTITY STATUS shown above. If the ENTITY STATUS is shown as SMALL or MICRO, verify whether entitlement to that entity status still applies.

If the ENTITY STATUS is the same as shown above, pay the TOTAL FEE(S) DUE shown above.

If the ENTITY STATUS is changed from that shown above, on PART B - FEE(S) TRANSMITTAL, complete section number 5 titled "Change in Entity Status (from status indicated above)".

For purposes of this notice, small entity fees are 1/2 the amount of undiscounted fees, and micro entity fees are 1/2 the amount of small entity fees.

II. PART B - FEE(S) TRANSMITTAL, or its equivalent, must be completed and returned to the United States Patent and Trademark Office (USPTO) with your ISSUE FEE and PUBLICATION FEE (if required). If you are charging the fee(s) to your deposit account, section "4b" of Part B - Fee(s) Transmittal should be completed and an extra copy of the form should be submitted. If an equivalent of Part B is filed, a request to reapply a previously paid issue fee must be clearly made, and delays in processing may occur due to the difficulty in recognizing the paper as an equivalent of Part B.

III. All communications regarding this application must give the application number. Please direct all communications prior to issuance to Mail Stop ISSUE FEE unless advised to the contrary.

IMPORTANT REMINDER: Utility patents issuing on applications filed on or after Dec. 12, 1980 may require payment of maintenance fees. It is patentee's responsibility to ensure timely payment of maintenance fees when due.

PTOL-85 (Rev. 02/11)

PART B - FEE(S) TRANSMITTAL

Complete and send this form, together with applicable fee(s), to: **Mail** **Mail Stop ISSUE FEE**
Commissioner for Patents
P.O. Box 1450
Alexandria, Virginia 22313-1450
or Fax (571)-273-2885

INSTRUCTIONS: This form should be used for transmitting the ISSUE FEE and PUBLICATION FEE (if required). Blocks 1 through 5 should be completed where appropriate. All further correspondence including the Patent, advance orders and notification of maintenance fees will be mailed to the current correspondence address as indicated unless corrected below or directed otherwise in Block 1, by (a) specifying a new correspondence address; and/or (b) indicating a separate "FEE ADDRESS" for maintenance fee notifications.

CURRENT CORRESPONDENCE ADDRESS (Note: Use Block 1 for any change of address)

26161 7590 09/16/2016
FISH & RICHARDSON P.C. (BO)
P.O. BOX 1022
MINNEAPOLIS, MN 55440-1022

Note: A certificate of mailing can only be used for domestic mailings of the Fee(s) Transmittal. This certificate cannot be used for any other accompanying papers. Each additional paper, such as an assignment or formal drawing, must have its own certificate of mailing or transmission.

Certificate of Mailing or Transmission
I hereby certify that this Fee(s) Transmittal is being deposited with the United States Postal Service with sufficient postage for first class mail in an envelope addressed to the Mail Stop ISSUE FEE address above, or being facsimile transmitted to the USPTO (571) 273-2885, on the date indicated below.

(Depositor's name)
(Signature)
(Date)

APPLICATION NO.	FILING DATE	FIRST NAMED INVENTOR	ATTORNEY DOCKET NO.	CONFIRMATION NO.
14/067,620	10/30/2013	Brian K. Maples	30171-0025002 / ITI-001	4288

TITLE OF INVENTION: Nicking and Extension Amplification Reaction for the Exponential Amplification of Nucleic Acids

APPLN. TYPE	ENTITY STATUS	ISSUE FEE DUE	PUBLICATION FEE DUE	PREV. PAID ISSUE FEE	TOTAL FEE(S) DUE	DATE DUE
nonprovisional	UNDISCOUNTED	$960	$0	$0	$960	12/16/2016

EXAMINER	ART UNIT	CLASS-SUBCLASS
BERTAGNA, ANGELA MARIE	1637	435-006120

1. Change of correspondence address or indication of "Fee Address" (37 CFR 1.363).
   - ❑ Change of correspondence address (or Change of Correspondence Address form PTO/SB/122) attached.
   - ❑ "Fee Address" indication (or "Fee Address" Indication form PTO/SB/47; Rev 03-02 or more recent) attached. **Use of a Customer Number is required.**

2. For printing on the patent front page, list
   (1) The names of up to 3 registered patent attorneys or agents OR, alternatively,
   (2) The name of a single firm (having as a member a registered attorney or agent) and the names of up to 2 registered patent attorneys or agents. If no name is listed, no name will be printed.

   1 ____________________
   2 ____________________
   3 ____________________

3. ASSIGNEE NAME AND RESIDENCE DATA TO BE PRINTED ON THE PATENT (print or type)

PLEASE NOTE: Unless an assignee is identified below, no assignee data will appear on the patent. If an assignee is identified below, the document has been filed for recordation as set forth in 37 CFR 3.11. Completion of this form is NOT a substitute for filing an assignment.

(A) NAME OF ASSIGNEE

(B) RESIDENCE: (CITY and STATE OR COUNTRY)

Please check the appropriate assignee category or categories (will not be printed on the patent) : ❑ Individual ❑ Corporation or other private group entity ❑ Government

4a. The following fee(s) are submitted:
- ❑ Issue Fee
- ❑ Publication Fee (No small entity discount permitted)
- ❑ Advance Order - # of Copies ____________

4b. Payment of Fee(s): **(Please first reapply any previously paid issue fee shown above)**
- ❑ A check is enclosed.
- ❑ Payment by credit card. Form PTO-2038 is attached.
- ❑ The director is hereby authorized to charge the required fee(s), any deficiency, or credits any overpayment, to Deposit Account Number ____________ (enclose an extra copy of this form).

5. **Change in Entity Status** (from status indicated above)
- ❑ Applicant certifying micro entity status. See 37 CFR 1.29
- ❑ Applicant asserting small entity status. See 37 CFR 1.27
- ❑ Applicant changing to regular undiscounted fee status.

NOTE: Absent a valid certification of Micro Entity Status (see forms PTO/SB/15A and 15B), issue fee payment in the micro entity amount will not be accepted at the risk of application abandonment.

NOTE: If the application was previously under micro entity status, checking this box will be taken to be a notification of loss of entitlement to micro entity status.

NOTE: Checking this box will be taken to be a notification of loss of entitlement to small or micro entity status, as applicable.

NOTE: This form must be signed in accordance with 37 CFR 1.31 and 1.33. See 37 CFR 1.4 for signature requirements and certifications.

Authorized Signature ____________________ Date ____________________

Typed or printed name ____________________ Registration No. ____________________

PTOL-85 Part B (10-13) Approved for use through 10/31/2013. OMB 0651-0033 U.S. Patent and Trademark Office; U.S. DEPARTMENT OF COMMERCE

UNITED STATES PATENT AND TRADEMARK OFFICE

UNITED STATES DEPARTMENT OF COMMERCE
United States Patent and Trademark Office
Address: COMMISSIONER FOR PATENTS
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Alexandria, Virginia 22313-1450
www.uspto.gov

APPLICATION NO.	FILING DATE	FIRST NAMED INVENTOR	ATTORNEY DOCKET NO.	CONFIRMATION NO.
14/067,620	10/30/2013	Brian K. Maples	30171-0025002 / ITI-001	4288

26161 7590 09/16/2016
FISH & RICHARDSON P.C. (BO)
P.O. BOX 1022
MINNEAPOLIS, MN 55440-1022

EXAMINER	
BERTAGNA, ANGELA MARIE	

ART UNIT	PAPER NUMBER
1637	

DATE MAILED: 09/16/2016

Determination of Patent Term Adjustment under 35 U.S.C. 154 (b)
(Applications filed on or after May 29, 2000)

The Office has discontinued providing a Patent Term Adjustment (PTA) calculation with the Notice of Allowance.

Section 1(h)(2) of the AIA Technical Corrections Act amended 35 U.S.C. 154(b)(3)(B)(i) to eliminate the requirement that the Office provide a patent term adjustment determination with the notice of allowance. See Revisions to Patent Term Adjustment, 78 Fed. Reg. 19416, 19417 (Apr. 1, 2013). Therefore, the Office is no longer providing an initial patent term adjustment determination with the notice of allowance. The Office will continue to provide a patent term adjustment determination with the Issue Notification Letter that is mailed to applicant approximately three weeks prior to the issue date of the patent, and will include the patent term adjustment on the patent. Any request for reconsideration of the patent term adjustment determination (or reinstatement of patent term adjustment) should follow the process outlined in 37 CFR 1.705.

Any questions regarding the Patent Term Extension or Adjustment determination should be directed to the Office of Patent Legal Administration at (571)-272-7702. Questions relating to issue and publication fee payments should be directed to the Customer Service Center of the Office of Patent Publication at 1-(888)-786-0101 or (571)-272-4200.

PTOL-85 (Rev. 02/11)

OMB Clearance and PRA Burden Statement for PTOL-85 Part B

The Paperwork Reduction Act (PRA) of 1995 requires Federal agencies to obtain Office of Management and Budget approval before requesting most types of information from the public. When OMB approves an agency request to collect information from the public, OMB (i) provides a valid OMB Control Number and expiration date for the agency to display on the instrument that will be used to collect the information and (ii) requires the agency to inform the public about the OMB Control Number's legal significance in accordance with 5 CFR 1320.5(b).

The information collected by PTOL-85 Part B is required by 37 CFR 1.311. The information is required to obtain or retain a benefit by the public which is to file (and by the USPTO to process) an application. Confidentiality is governed by 35 U.S.C. 122 and 37 CFR 1.14. This collection is estimated to take 12 minutes to complete, including gathering, preparing, and submitting the completed application form to the USPTO. Time will vary depending upon the individual case. Any comments on the amount of time you require to complete this form and/or suggestions for reducing this burden, should be sent to the Chief Information Officer, U.S. Patent and Trademark Office, U.S. Department of Commerce, P.O. Box 1450, Alexandria, Virginia 22313-1450. DO NOT SEND FEES OR COMPLETED FORMS TO THIS ADDRESS. SEND TO: Commissioner for Patents, P.O. Box 1450, Alexandria, Virginia 22313-1450. Under the Paperwork Reduction Act of 1995, no persons are required to respond to a collection of information unless it displays a valid OMB control number.

Privacy Act Statement

The Privacy Act of 1974 (P.L. 93-579) requires that you be given certain information in connection with your submission of the attached form related to a patent application or patent. Accordingly, pursuant to the requirements of the Act, please be advised that: (1) the general authority for the collection of this information is 35 U.S.C. 2(b)(2); (2) furnishing of the information solicited is voluntary; and (3) the principal purpose for which the information is used by the U.S. Patent and Trademark Office is to process and/or examine your submission related to a patent application or patent. If you do not furnish the requested information, the U.S. Patent and Trademark Office may not be able to process and/or examine your submission, which may result in termination of proceedings or abandonment of the application or expiration of the patent.

The information provided by you in this form will be subject to the following routine uses:

1. The information on this form will be treated confidentially to the extent allowed under the Freedom of Information Act (5 U.S.C. 552) and the Privacy Act (5 U.S.C 552a). Records from this system of records may be disclosed to the Department of Justice to determine whether disclosure of these records is required by the Freedom of Information Act.
2. A record from this system of records may be disclosed, as a routine use, in the course of presenting evidence to a court, magistrate, or administrative tribunal, including disclosures to opposing counsel in the course of settlement negotiations.
3. A record in this system of records may be disclosed, as a routine use, to a Member of Congress submitting a request involving an individual, to whom the record pertains, when the individual has requested assistance from the Member with respect to the subject matter of the record.
4. A record in this system of records may be disclosed, as a routine use, to a contractor of the Agency having need for the information in order to perform a contract. Recipients of information shall be required to comply with the requirements of the Privacy Act of 1974, as amended, pursuant to 5 U.S.C. 552a(m).
5. A record related to an International Application filed under the Patent Cooperation Treaty in this system of records may be disclosed, as a routine use, to the International Bureau of the World Intellectual Property Organization, pursuant to the Patent Cooperation Treaty.
6. A record in this system of records may be disclosed, as a routine use, to another federal agency for purposes of National Security review (35 U.S.C. 181) and for review pursuant to the Atomic Energy Act (42 U.S.C. 218(c)).
7. A record from this system of records may be disclosed, as a routine use, to the Administrator, General Services, or his/her designee, during an inspection of records conducted by GSA as part of that agency's responsibility to recommend improvements in records management practices and programs, under authority of 44 U.S.C. 2904 and 2906. Such disclosure shall be made in accordance with the GSA regulations governing inspection of records for this purpose, and any other relevant (i.e., GSA or Commerce) directive. Such disclosure shall not be used to make determinations about individuals.
8. A record from this system of records may be disclosed, as a routine use, to the public after either publication of the application pursuant to 35 U.S.C. 122(b) or issuance of a patent pursuant to 35 U.S.C. 151. Further, a record may be disclosed, subject to the limitations of 37 CFR 1.14, as a routine use, to the public if the record was filed in an application which became abandoned or in which the proceedings were terminated and which application is referenced by either a published application, an application open to public inspection or an issued patent.
9. A record from this system of records may be disclosed, as a routine use, to a Federal, State, or local law enforcement agency, if the USPTO becomes aware of a violation or potential violation of law or regulation.

Examiner-Initiated Interview Summary	**Application No.** 14/067,620	**Applicant(s)** MAPLES ET AL.	
	Examiner Angela M. Bertagna	**Art Unit** 1637	

All participants (applicant, applicant's representative, PTO personnel):

(1) Angela M. Bertagna (PTO).

(2) Ian Lodovice (Applicant's representative).

(3) Belinda Lew (Applicant's representative).

(4) Jay Fister (Applicant's representative).

Date of Interview: 26 August 2016.

Type: ☒ Telephonic ☐ Video Conference
☐ Personal [copy given to: ☐ applicant ☐ applicant's representative]

Exhibit shown or demonstration conducted: ☐ Yes ☒ No.
If Yes, brief description: _____.

Issues Discussed ☐101 ☒112 ☐102 ☐103 ☒Others
(For each of the checked box(es) above, please describe below the issue and detailed description of the discussion)

Claim(s) discussed: 67-83,85-88,90,92 and 95-107.

Identification of prior art discussed: N/A.

Substance of Interview
(For each issue discussed, provide a detailed description and indicate if agreement was reached. Some topics may include: identification or clarification of a reference or a portion thereof, claim interpretation, proposed amendments, arguments of any applied references etc...)

See Continuation Sheet.

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Examiner recordation instructions: Examiners must summarize the substance of any interview of record. A complete and proper recordation of the substance of an interview should include the items listed in MPEP 713.04 for complete and proper recordation including the identification of the general thrust of each argument or issue discussed, a general indication of any other pertinent matters discussed regarding patentability and the general results or outcome of the interview, to include an indication as to whether or not agreement was reached on the issues raised.

☒ Attachment

/Angela M. Bertagna/
Primary Examiner, Art Unit 1637

Continuation of Substance of Interview including description of the general nature of what was agreed to if an agreement was reached, or any other comments: The examiner stated that the claims contain allowable subject matter in view of the requirement for use of an amplification reaction mixture lacking bumper primers and proposed amending step (c) of claims 67, 95, and 107 such that the amplification step clearly excludes the use of bumper primers. We discussed possible claim language, such as "without the assistance of bumper primers", to add to step (c) of claims 67, 95, and 107. The examiner also proposed typographical and/or grammatical changes to claims 67, 90, 95, and 107. We also discussed claims 68-70. The examiner stated that these claims appear to state inherent features of the method of claim 67 and may not be further limiting for this reason. We discussed possible amendments that could be made to these claims, including requiring a particular target nucleic acid. We also discussed canceling or amending claim 87 since it does not appear to be further limiting.The examiner further stated that all withdrawn claims would be rejoined, and asked for terminal disclaimers to be filed over Application Serial Nos. 14067623, 11778018, and 12173020. The examiner additionally proposed canceling one of claims 73 and 83 since these claims appear to be duplicates. Lastly, we discussed possible amendments that could be made to independent claims 67, 95, and 107 so that they will not have the same scope as any of the claims of the aforementioned '623 application.

Applicant's representatives stated that the proposal would be considered and an amendment incorporating the proposed changes would be faxed to the examiner. This fax was received on August 29, 2016 and is attached. The examiner proposed additional changes after reviewing the fax on August 30, 2016 to Ian Lodovice. A second proposal was received by fax on August 30, 2016 and is also attached. The examiner stated that this second proposal was acceptable. Applicant's representative (Ian Lodovice) stated that the fax of August 30, 2016 would be filed as a supplemental amendment together with the required terminal disclaimers.

Notice of Allowability	Application No. 14/067,620	Applicant(s) MAPLES ET AL.	
	Examiner Angela M. Bertagna	Art Unit 1637	AIA (First Inventor to File) Status No

-- The MAILING DATE of this communication appears on the cover sheet with the correspondence address--

All claims being allowable, PROSECUTION ON THE MERITS IS (OR REMAINS) CLOSED in this application. If not included herewith (or previously mailed), a Notice of Allowance (PTOL-85) or other appropriate communication will be mailed in due course. **THIS NOTICE OF ALLOWABILITY IS NOT A GRANT OF PATENT RIGHTS.** This application is subject to withdrawal from issue at the initiative of the Office or upon petition by the applicant. See 37 CFR 1.313 and MPEP 1308.

1. ☒ This communication is responsive to *the response filed on September 2, 2016*.
   ☐ A declaration(s)/affidavit(s) under **37 CFR 1.130(b)** was/were filed on______.
2. ☐ An election was made by the applicant in response to a restriction requirement set forth during the interview on _____; the restriction requirement and election have been incorporated into this action.
3. ☒ The allowed claim(s) is/are *67-83,85,86,88,90,92 and 95-107*. As a result of the allowed claim(s), you may be eligible to benefit from the **Patent Prosecution Highway** program at a participating intellectual property office for the corresponding application. For more information, please see http://www.uspto.gov/patents/init_events/pph/index.jsp or send an inquiry to PPHfeedback@uspto.gov.
4. ☐ Acknowledgment is made of a claim for foreign priority under 35 U.S.C. § 119(a)-(d) or (f).

   Certified copies:

   a) ☐ All b) ☐ Some *c) ☐ None of the:
   1. ☐ Certified copies of the priority documents have been received.
   2. ☐ Certified copies of the priority documents have been received in Application No. _____ .
   3. ☐ Copies of the certified copies of the priority documents have been received in this national stage application from the International Bureau (PCT Rule 17.2(a)).

   * Certified copies not received: _____.

Applicant has THREE MONTHS FROM THE "MAILING DATE" of this communication to file a reply complying with the requirements noted below. Failure to timely comply will result in ABANDONMENT of this application.
THIS THREE-MONTH PERIOD IS NOT EXTENDABLE.

5. ☐ CORRECTED DRAWINGS (as "replacement sheets") must be submitted.
   ☐ including changes required by the attached Examiner's Amendment / Comment or in the Office action of Paper No./Mail Date _____.

Identifying indicia such as the application number (see 37 CFR 1.84(c)) should be written on the drawings in the front (not the back) of each sheet. Replacement sheet(s) should be labeled as such in the header according to 37 CFR 1.121(d).

6. ☐ DEPOSIT OF and/or INFORMATION about the deposit of BIOLOGICAL MATERIAL must be submitted. Note the attached Examiner's comment regarding REQUIREMENT FOR THE DEPOSIT OF BIOLOGICAL MATERIAL.

Attachment(s)

1. ☐ Notice of References Cited (PTO-892)
2. ☒ Information Disclosure Statements (PTO/SB/08), Paper No./Mail Date _____
3. ☐ Examiner's Comment Regarding Requirement for Deposit of Biological Material
4. ☒ Interview Summary (PTO-413), Paper No./Mail Date _____ .
5. ☒ Examiner's Amendment/Comment
6. ☐ Examiner's Statement of Reasons for Allowance
7. ☐ Other _____.

/Angela M. Bertagna/
Primary Examiner, Art Unit 1637

DETAILED ACTION

Notice of Pre-AIA or AIA Status

1. The present application is being examined under the pre-AIA first to invent provisions.

Response to Arguments

2. The previously made rejection of claim 107 under pre-AIA 35 U.S.C. 112, first paragraph (new matter) has been withdrawn as being obviated by the claim amendments. This argument is also made by Applicant (see page 10 of the response filed on August 11, 2016).

The previously made rejections of claims 67-73, 76, 77, 80, 83, 85-88, 90-92, and 96-107 under pre-AIA 35 U.S.C. 103(a) citing Wick and Kong (claims 67-73, 76, 77, 80, 83, 85-88, 90-92, and 96-98) and Wick, Kong, and Yao (claims 99-107) have been withdrawn in view of the amendments to claim 67, which require conducting the amplification reaction without the assistance of bumper primers and detecting amplification product within ten minutes of beginning the amplification reaction. The only reference disclosing short amplification times is Yao, but this reference only describes short amplification times when bumper primers are used. Therefore, the ordinary artisan would have had neither a motivation nor a reasonable expectation of success in obtaining sufficient amplification product for detection within ten minutes when the amplification reaction is conducted without bumper primers as required by the claims. This argument is also made by Applicant (see page 13, last paragraph - page 14, first paragraph of the response filed on August 11, 2016).

Applicant's additional arguments filed on August 11, 2016 have been considered, but they are moot since the rejections under § 103(a) have been withdrawn for the above reasons.

The previously made provisional obviousness-type double patenting rejections have been withdrawn in view of Applicant's submission of terminal disclaimers on September 2, 2016.

Terminal Disclaimer

3. The terminal disclaimer filed on September 2, 2016 disclaiming the terminal portion of any patent granted on this application which would extend beyond the expiration date of any patent granted on (1) Application Serial No. 14/067,623; (2) Application Serial No. 12/173,020; and (3) Application Serial No. 11/778,018; has been reviewed and is accepted. The terminal disclaimer has been recorded.

Election/Restrictions

4. Claim 67 is allowable. Claims 74, 75, 78, 79, 81, 82, and 95, previously withdrawn from consideration as a result of an election of species requirement, require all of the limitations of an allowable claim. Pursuant to the procedures set forth in MPEP § 821.04(a), **the election of species requirement between the different sample type and different target nucleic acids as set forth in the Office action mailed on March 4, 2014, is hereby withdrawn** and claims 74, 75, 78, 79, 81, 82, and 95 are hereby rejoined and fully examined for patentability under 37 CFR 1.104. In view of the withdrawal of the restriction requirement, Applicant is advised that if any claim presented in a continuation or divisional application is anticipated by, or includes all the limitations of, a claim that is allowable in the present application, such claim may be subject to provisional statutory and/or nonstatutory double patenting rejections over the claims of the instant application. Once the restriction requirement is withdrawn, the provisions of 35 U.S.C.

121 are no longer applicable. See *In re Ziegler*, 443 F.2d 1211, 1215, 170 USPQ 129, 131-32 (CCPA 1971). See also MPEP § 804.01.

Allowable Subject Matter

5. Claims 67-83, 85, 86, 88, 90, 92, and 95-107 are allowed.

Conclusion

6. Any inquiry concerning this communication or earlier communications from the examiner should be directed to Angela M. Bertagna whose telephone number is (571)272-8291. The examiner can normally be reached on Monday-Friday, 9-5.

If attempts to reach the examiner by telephone are unsuccessful, the examiner's supervisor, Gary Benzion can be reached on (571)272-0782. The fax phone number for the organization where this application or proceeding is assigned is 571-273-8300.

Information regarding the status of an application may be obtained from the Patent Application Information Retrieval (PAIR) system. Status information for published applications may be obtained from either Private PAIR or Public PAIR. Status information for unpublished applications is available through Private PAIR only. For more information about the PAIR system, see http://pair-direct.uspto.gov. Should you have questions on access to the Private PAIR system, contact the Electronic Business Center (EBC) at 866-217-9197 (toll-free). If you would like assistance from a USPTO Customer Service Representative or access to the automated information system, call 800-786-9199 (IN USA OR CANADA) or 571-272-1000.

/Angela M. Bertagna/
Primary Examiner, Art Unit 1637

Examiner-Initiated Interview Summary	**Application No.** 14/067,620	**Applicant(s)** MAPLES ET AL.	
	Examiner Angela M. Bertagna	**Art Unit** 1637	

All participants (applicant, applicant's representative, PTO personnel):

(1) Angela M. Bertagna (PTO).

(2) Ian Lodovice (Applicant's representative).

(3) Belinda Lew (Applicant's representative).

(4) Jay Fister (Applicant's representative).

Date of Interview: 26 August 2016.

Type: ☒ Telephonic ☐ Video Conference
☐ Personal [copy given to: ☐ applicant ☐ applicant's representative]

Exhibit shown or demonstration conducted: ☐ Yes ☒ No.
If Yes, brief description: _____.

Issues Discussed ☐101 ☒112 ☐102 ☐103 ☒Others
(For each of the checked box(es) above, please describe below the issue and detailed description of the discussion)

Claim(s) discussed: 67-83,85-88,90,92 and 95-107.

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☒ Attachment

/Angela M. Bertagna/ Primary Examiner, Art Unit 1637	

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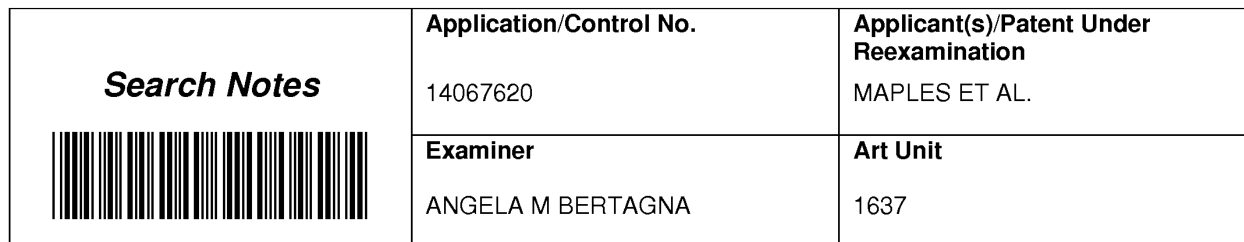

Search Notes	Application/Control No.	Applicant(s)/Patent Under Reexamination
	14067620	MAPLES ET AL.
	Examiner	**Art Unit**
	ANGELA M BERTAGNA	1637

CPC- SEARCHED

Symbol	Date	Examiner

CPC COMBINATION SETS - SEARCHED

Symbol	Date	Examiner

US CLASSIFICATION SEARCHED

Class	Subclass	Date	Examiner

SEARCH NOTES

Search Notes	Date	Examiner
searched all inventors by name	10/10/2014	amb
EAST search history attached	10/10/2014; 10/14/2014	amb
Google Scholar (search terms included "nicking", "isothermal", and "RNA")	10/10/2014; 10/14/2014	amb
reviewed related cases - 14067623 & 11778018	10/10/2014	amb
updated search	2/18/15; 2/19/15	amb
updated search	2/4/2016	amb
updated search	8/24/2016; 8/26/2016; 9/8/2016	amb

INTERFERENCE SEARCH

US Class/ CPC Symbol	US Subclass / CPC Group	Date	Examiner
searched USPGPUBS		9/8/2016	amb

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

First Named Inventor	:	Brian K. Maples	Art Unit	:	1637
Serial No.	:	14/067,620	Examiner	:	Angela M. Bertagna
Filed	:	October 30, 2013	Conf. No.	:	4288

Title : NICKING AND EXTENSION AMPLIFICATION REACTION FOR THE EXPONENTIAL AMPLIFICATION OF NUCLEIC ACIDS

ATTN: Examiner Bertanga

RE: Updated Proposed Amendment (revised amendment to claims 71-77 and 95)

FAX: 571-273-8291

PAGE 1/10 * RCVD AT 8/30/2016 3:46:42 PM [Eastern Daylight Time] * SVR:W-PTOFAX-001/13 * DNIS:2738291 * CSID:6175428906 * DURATION (mm-ss):01-50

List of Claims

1. - 66. (Canceled)

67. (Currently Amended) A method of amplifying a target polynucleotide sequence ~~of a target nucleic acid present in a sample obtained from an animal~~, the method comprising:
(a) obtaining, from an animal, plant or food, a sample comprising a target nucleic acid, the target nucleic acid comprising the target polynucleotide sequence.

~~(a)~~ (b) ~~preparing,~~ without first subjecting the target nucleic acid to a thermal denaturation step associated with amplification of the target polynucleotide sequence, combining, in a single step, the obtained sample directly with an amplification reagent mixture or diluting the obtained sample and combining, in a single step, the diluted sample with ~~a~~ an amplification reagent mixture, in either case, the amplification reagent mixture being free of bumper primers and comprising:

(i) ~~the target nucleic acid comprising the target polynucleotide sequence,~~

~~(ii)~~ a polymerase,

~~(iii)~~ (ii) a nicking enzyme,

~~(iv)~~ (iii) a first oligonucleotide comprising a nicking site and a nicking enzyme binding site, and

~~(v)~~ (iv) a second oligonucleotide comprising a nicking site and a nicking enzyme binding site, ~~and~~

~~(b)~~ (c) subjecting the reaction mixture formed by the step of combining to essentially isothermal conditions to amplify the target polynucleotide sequence without the assistance of bumper primers, and

(d) detecting the amplified target polynucleotide sequence in real time within 10 minutes of subjecting the reaction mixture to essentially isothermal conditions.

68. (Currently Amended) The method of claim 67, wherein the sample is obtained from an animal and the target polynucleotide sequence is amplified from steps comprising:

(a) forming a first duplex comprising the target polynucleotide sequence and the first oligonucleotide;

(b) extending, using the polymerase, the first oligonucleotide along the target polynucleotide sequence to form an extended first oligonucleotide comprising a sequence complementary to the second oligonucleotide;

(c) forming a second duplex comprising the second oligonucleotide and the extended first oligonucleotide;

(d) extending, using the polymerase, the second oligonucleotide along the extended first oligonucleotide to form a third duplex comprising an extended second oligonucleotide comprising a sequence complementary to the first oligonucleotide and a first double-stranded nicking enzyme binding site;

(e) nicking, with the nicking enzyme, the first nicking site on the third duplex to produce a fourth duplex comprising the extended second oligonucleotide and a fragment of the extended first oligonucleotide; and

(f) extending, using the polymerase, the fragment of the extended first oligonucleotide along the extended second oligonucleotide of the fourth duplex to produce a double-stranded nucleic acid product ~~and~~ containing a second double-stranded nicking enzyme binding site.

69. (Currently Amended) The method of claim 68, wherein sample is obtained from an animal and the double-stranded nucleic acid product comprises:

i) a first strand and a second strand, wherein the first strand comprises a first polynucleotide sequence corresponding to the target polynucleotide sequence and the second strand comprises a second polynucleotide sequence complementary to the target polynucleotide sequence, and

ii) first and second double-stranded nicking sites spaced apart by the target polynucleotide sequence.

70. (Currently Amended) The method of claim 68, further comprising the steps of:

~~a)~~ g) nicking, using the nicking enzyme, the first nicking site of the double-stranded nucleic acid product to produce a fifth duplex comprising a first polynucleotide sequence corresponding to the target polynucleotide sequence and a fragment of the first oligonucleotide, and nicking, using the nicking enzyme, the second nicking site of the double-stranded nucleic

PAGE 3/10 * RCVD AT 8/30/2016 3:46:42 PM [Eastern Daylight Time] * SVR:W-PTOFAX-001/13 * DNIS:2738291 * CSID:6175428906 * DURATION (mm-ss):01-50

acid product to produce a sixth duplex comprising a second polynucleotide sequence complementary to the target polynucleotide sequence and a fragment of the second oligonucleotide;

~~b)~~ h) extending, using the polymerase, the fragment of the first oligonucleotide along the first polynucleotide sequence of the fifth duplex to produce a first double stranded product comprising a copy of the nicking site and a copy of the first polynucleotide sequence and extending, using the polymerase, the fragment of the second oligonucleotide along the second polynucleotide sequence of the sixth duplex to produce a second double stranded product comprising a copy of the nicking site and a copy of the second polynucleotide sequence; and

~~c)~~ i) nicking, using the nicking enzyme, the copy of the nicking site of the first double stranded product to release a copy of the first polynucleotide sequence and nicking, using the nicking enzyme, the copy of the nicking site of the second double stranded product to release a copy of the second polynucleotide sequence.

71. (Currently Amended) The method of claim 67, wherein the sample is obtained from an animal and the animal is a human, the target nucleic acid is a target nucleic acid of a human pathogen, and the sample is obtained from the mucus, sputum, or saliva of the human.

72. (Currently Amended) The method of claim 67, wherein the sample is obtained from a human and the target nucleic acid is ~~obtained from~~ a target nucleic acid of a human ~~an animal~~ pathogen.

73. (Currently Amended) The method of claim 72, wherein the human ~~animal~~ pathogen is a single-stranded DNA virus, double-stranded DNA virus, or single-stranded RNA virus.

74. (Withdrawn, Currently Amended) The method of claim 72, wherein the human ~~animal~~ pathogen is a bacterium.

75. (Withdrawn, Currently Amended) The method of claim 72, wherein the human ~~animal~~ pathogen contains spores and the target polynucleotide is amplified from the spores without the need for lysis of the spores.

76. (Currently Amended) The method of claim 67, wherein the animal is human and the sample is obtained from an animal and is ~~obtained from the~~ blood, ~~bone marrow,~~ mucus, ~~lymph, hard tissues, biopsies,~~ sputum, saliva, tears, feces ~~faeces~~ or urine of the animal of the human.

77. (Currently Amended) The method of claim 76, wherein the sample is obtained from an animal and is ~~obtained from~~ the mucus, sputum, or saliva of the human~~animal~~.

78. (Withdrawn) The method of claim 67, wherein the target nucleic acid is double-stranded DNA.

79. (Withdrawn) The method of claim 67, wherein the target nucleic acid is single-stranded DNA.

80. (Previously Presented) The method of claim 67, wherein the target nucleic acid is RNA.

81. (Withdrawn) The method of claim 67, wherein the target nucleic acid is selected from the group consisting of genomic DNA, plasmid DNA, viral DNA, mitochondrial DNA, cDNA, synthetic double-stranded DNA and synthetic single-stranded DNA.

82. (Withdrawn) The method of claim 81, wherein the target nucleic acid is genomic DNA.

83. (Previously Presented) The method of claim 67, wherein the target nucleic acid is viral DNA or viral RNA.

84. (Canceled)

85. (Previously Presented) The method of claim 67, wherein the nicking enzyme is Nt.BstNBI.

86. (Previously Presented) The method of claim 67, wherein the nicking enzyme does not nick within the target polynucleotide sequence.

87. (Canceled) ~~The method of claim 67, wherein amplification of the target polynucleotide sequence is performed without the use of temperature cycling.~~

88. (Previously Presented) The method of claim 67, wherein amplification of the target polynucleotide sequence is performed at about 55°C-59°C.

89. (Canceled)

90. (Currently Amended) The method of claim ~~68~~ 67, ~~which~~ wherein the step of amplifying the target polynucleotide sequence is performed at a temperature higher than the melting temperature of the first oligonucleotide/target polynucleotide sequence complex.

91. (Canceled)

92. (Currently Amended) The method of claim 67 ~~91~~, wherein the amplification product is detected by a detection method selected from the group consisting of ~~gel electrophoresis, mass spectrometry~~, fluorescence, intercalating dye detection, fluorescence resonance energy transfer (FRET), molecular beacon detection, ~~surface capture, capillary electrophoresis,~~ and incorporation of labeled nucleotides ~~to allow detection by capture, fluorescence polarization, and lateral flow capture, or a combination thereof~~.

93. (Canceled)

94. (Canceled)

95. (Withdrawn, Currently Amended) A method of amplifying a target polynucleotide sequence ~~of genomic DNA present in a sample obtained from an animal~~, the method comprising:

(a) obtaining, from a human, plant, or food, a sample containing genomic DNA, the genomic DNA comprising a target nucleic acid, the target nucleic acid comprising the target polynucleotide sequence,

(b) ~~preparing,~~ without first subjecting the genomic DNA to a thermal denaturation step associated with amplification of the target polynucleotide sequence, combining, in a single step, the obtained sample directly with an amplification reagent mixture or diluting the obtained sample and combining, in a single step, the diluted sample with ~~a~~ an amplification reagent mixture, in either case, the amplification reagent mixture being free of bumper primers and comprising:

(i) ~~the genomic DNA comprising the target polynucleotide sequence,~~

~~(ii)~~ a polymerase,

~~(iii)~~ (ii) a nicking enzyme,

~~(iv)~~ (iii) a first oligonucleotide comprising a nicking site and a nicking enzyme binding site, ~~and~~

~~(v)~~ (iv) a second oligonucleotide comprising a nicking site and a nicking enzyme binding site, and

~~b)~~ (c) subjecting the reaction mixture formed by the step of combining to essentially isothermal conditions to amplify the target polynucleotide sequence without the assistance of bumper primers, and

(d) detecting the amplified target polynucleotide sequence in real time within 10 minutes of subjecting the reaction mixture to essentially isothermal conditions.

PAGE 7/10 * RCVD AT 8/30/2016 3:46:42 PM [Eastern Daylight Time] * SVR:W-PTOFAX-001/13 * DNIS:2738291 * CSID:6175428906 * DURATION (mm-ss):01-50

96. (Previously Presented) The method of claim 67, wherein the target polynucleotide sequence is amplified about 1E+8-fold.

97. (Previously Presented) The method of claim 67, wherein the target polynucleotide sequence is amplified about 3E+9-fold.

98. (Previously Presented) The method of claim 67, wherein the target polynucleotide sequence is amplified about 7E+10-fold.

99. (Previously Presented) The method of claim 67, wherein amplification of the target polynucleotide sequence is performed at a constant temperature for about 1 to 12 minutes.

100. (Previously Presented) The method of claim 67, wherein amplification of the target polynucleotide sequence is performed at a constant temperature for about 1 to 10 minutes.

101. (Previously Presented) The method of claim 67, wherein amplification of the target polynucleotide sequence is performed at a constant temperature for about 1 to 8 minutes.

102. (Previously Presented) The method of claim 67, wherein amplification of the target polynucleotide sequence is performed at a constant temperature for about 1 to 5 minutes.

103. (Previously Presented) The method of claim 67, wherein amplification of the target polynucleotide sequence is performed at a constant temperature for about 1 to 2.5 minutes.

104. (Previously Presented) The method of claim 67, wherein amplification of the target polynucleotide sequence is performed at a constant temperature for about 2.5 to 5 minutes.

105. (Previously Presented) The method of claim 67, wherein amplification of the target polynucleotide sequence is performed at a constant temperature for about 2.5 to 8 minutes.

106. (Previously Presented) The method of claim 67, wherein amplification of the target polynucleotide sequence is performed at a constant temperature for about 2.5 to 10 minutes.

107. (Currently Amended) A method of amplifying a target polynucleotide sequence ~~of a target nucleic acid present in a sample obtained from an animal~~, the method comprising:

(a) obtaining, from an animal, plant or food, a sample comprising a target nucleic acid, the target nucleic acid comprising the target polynucleotide sequence,

~~(a)~~ (b) ~~preparing,~~ without first subjecting the target nucleic acid to a thermal denaturation step associated with amplification of the target polynucleotide sequence, combining, in a single step, the obtained sample directly with an amplification reagent mixture or diluting the obtained sample and combining, in a single step, the diluted sample with ~~a~~ an amplification reagent mixture, in either case, the amplification reagent mixture being free of bumper primers and comprising:

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~~(iv)~~ (iii) a first oligonucleotide comprising a nicking site and a nicking enzyme binding site, and

~~(v)~~ (iv) a second oligonucleotide comprising a nicking site and a nicking enzyme binding site, ~~and~~

~~(b)~~ (c) subjecting the reaction mixture formed by the step of combining to essentially isothermal conditions to amplify the target polynucleotide sequence without the assistance of bumper primers,

(d) detecting the amplified target polynucleotide sequence in real time within 10 minutes of subjecting the reaction mixture to essentially isothermal conditions, and

wherein the target polynucleotide sequence is amplified about 1E+8-fold in less than about ~~1 to~~ 12 minutes.

Attorney Docket No.: 30171-0025002 / Client Ref: ITI-001

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

First Named Inventor	:	Brian K. Maples	Art Unit	:	1637
Serial No.	:	14/067,620	Examiner	:	Angela M. Bertagna
Filed	:	October 30, 2013	Conf. No.	:	4288

Title : NICKING AND EXTENSION AMPLIFICATION REACTION FOR THE EXPONENTIAL AMPLIFICATION OF NUCLEIC ACIDS

ATTN: Examiner Bertanga

RE: Proposed Amendment following Examienr Interview of August 26, 2016

FAX: 571-273-8291

Listing of Claims

1. - 66. (Canceled)

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~~(v)~~ (iv) a second oligonucleotide comprising a nicking site and a nicking enzyme binding site, ~~and~~

~~(b)~~ (c) subjecting the reaction mixture formed by the step of combining to essentially isothermal conditions to amplify the target polynucleotide sequence without the assistance of bumper primers, and

(d) detecting the amplified target polynucleotide sequence in real time within 10 minutes of subjecting the reaction mixture to essentially isothermal conditions.

68. (Currently amended) The method of claim 67, wherein the sample is obtained from an animal and the target polynucleotide sequence is amplified from steps comprising:

(a) forming a first duplex comprising the target polynucleotide sequence and the first oligonucleotide;

(b) extending, using the polymerase, the first oligonucleotide along the target polynucleotide sequence to form an extended first oligonucleotide comprising a sequence complementary to the second oligonucleotide;

(c) forming a second duplex comprising the second oligonucleotide and the extended first oligonucleotide;

(d) extending, using the polymerase, the second oligonucleotide along the extended first oligonucleotide to form a third duplex comprising an extended second oligonucleotide comprising a sequence complementary to the first oligonucleotide and a first double-stranded nicking enzyme binding site;

(e) nicking, with the nicking enzyme, the first nicking site on the third duplex to produce a fourth duplex comprising the extended second oligonucleotide and a fragment of the extended first oligonucleotide; and

(f) extending, using the polymerase, the fragment of the extended first oligonucleotide along the extended second oligonucleotide of the fourth duplex to produce a double-stranded nucleic acid product ~~and~~ containing a second double-stranded nicking enzyme binding site.

69. (Currently amended) The method of claim 68, wherein sample is obtained from an animal and the double-stranded nucleic acid product comprises:

i) a first strand and a second strand, wherein the first strand comprises a first polynucleotide sequence corresponding to the target polynucleotide sequence and the second strand comprises a second polynucleotide sequence complementary to the target polynucleotide sequence, and

ii) first and second double-stranded nicking sites spaced apart by the target polynucleotide sequence.

70. (Currently amended) The method of claim 68, further comprising the steps of:

~~a)~~ g) nicking, using the nicking enzyme, the first nicking site of the double-stranded nucleic acid product to produce a fifth duplex comprising a first polynucleotide sequence

corresponding to the target polynucleotide sequence and a fragment of the first oligonucleotide, and nicking, using the nicking enzyme, the second nicking site of the double-stranded nucleic acid product to produce a sixth duplex comprising a second polynucleotide sequence complementary to the target polynucleotide sequence and a fragment of the second oligonucleotide;

~~b)~~ h) extending, using the polymerase, the fragment of the first oligonucleotide along the first polynucleotide sequence of the fifth duplex to produce a first double stranded product comprising a copy of the nicking site and a copy of the first polynucleotide sequence and extending, using the polymerase, the fragment of the second oligonucleotide along the second polynucleotide sequence of the sixth duplex to produce a second double stranded product comprising a copy of the nicking site and a copy of the second polynucleotide sequence; and

~~c)~~ i) nicking, using the nicking enzyme, the copy of the nicking site of the first double stranded product to release a copy of the first polynucleotide sequence and nicking, using the nicking enzyme, the copy of the nicking site of the second double stranded product to release a copy of the second polynucleotide sequence.

71. (Currently amended) The method of claim 67, wherein the sample is obtained from an animal and the animal is a human.

72. (Currently amended) The method of claim 67, wherein the sample is obtained from an animal and the target nucleic acid is ~~obtained from~~ a target nucleic acid of an animal pathogen.

73. (Previously Presented) The method of claim 72, wherein the animal pathogen is a single-stranded DNA virus, double-stranded DNA virus, or single-stranded RNA virus.

74. (Withdrawn) The method of claim 72, wherein the animal pathogen is a bacterium.

75. (Withdrawn) The method of claim 72, wherein the animal pathogen contains spores and the target polynucleotide is amplified from the spores without the need for lysis of the spores.

76. (Currently Amended) The method of claim 67, wherein the sample is obtained from an animal and is ~~obtained from the~~ blood, ~~bone marrow,~~ mucus, ~~lymph, hard tissues, biopsies,~~ sputum, saliva, tears, feces ~~faeces~~ or urine of the animal.

77. (Currently Amended) The method of claim 76, wherein the sample is obtained from an animal and is ~~obtained from the~~ mucus, sputum, or saliva of the animal.

78. (Withdrawn) The method of claim 67, wherein the target nucleic acid is double-stranded DNA.

79. (Withdrawn) The method of claim 67, wherein the target nucleic acid is single-stranded DNA.

80. (Previously Presented) The method of claim 67, wherein the target nucleic acid is RNA.

81. (Withdrawn) The method of claim 67, wherein the target nucleic acid is selected from the group consisting of genomic DNA, plasmid DNA, viral DNA, mitochondrial DNA, cDNA, synthetic double-stranded DNA and synthetic single-stranded DNA.

82. (Withdrawn) The method of claim 81, wherein the target nucleic acid is genomic DNA.

83. (Previously Presented) The method of claim 67, wherein the target nucleic acid is viral DNA or viral RNA.

84. (Canceled)

85. (Previously Presented) The method of claim 67, wherein the nicking enzyme is Nt.BstNBI.

86. (Previously Presented) The method of claim 67, wherein the nicking enzyme does not nick within the target polynucleotide sequence.

87. (Canceled) ~~The method of claim 67, wherein amplification of the target polynucleotide sequence is performed without the use of temperature cycling.~~

88. (Previously Presented) The method of claim 67, wherein amplification of the target polynucleotide sequence is performed at about 55°C-59°C.

89. (Canceled)

90. (Currently amended) The method of claim ~~68~~ 67, ~~which~~ wherein the step of amplifying the target polynucleotide sequence is performed at a temperature higher than the melting temperature of the first oligonucleotide/target polynucleotide sequence complex.

91. (Canceled)

92. (Currently Amended) The method of claim 67 ~~91~~, wherein the amplification product is detected by a detection method selected from the group consisting of ~~gel electrophoresis, mass spectrometry~~, fluorescence, intercalating dye detection, fluorescence resonance energy transfer (FRET), molecular beacon detection, ~~surface capture, capillary electrophoresis,~~ and incorporation of labeled nucleotides ~~to allow detection by capture, fluorescence polarization, and lateral flow capture, or a combination thereof~~.

93. (Canceled)

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94. (Canceled)

95. (Withdrawn, Currently Amended) A method of amplifying a target polynucleotide sequence ~~of genomic DNA present in a sample obtained from an animal~~, the method comprising:

(a) obtaining, from an animal, plant, or food, a sample containing genomic DNA, the genomic DNA comprising a target nucleic acid, the target nucleic acid comprising the target polynucleotide sequence,

(b) ~~preparing,~~ without first subjecting the genomic DNA to a thermal denaturation step associated with amplification of the target polynucleotide sequence, combining, in a single step, the obtained sample directly with an amplification reagent mixture or diluting the obtained sample and combining, in a single step, the diluted sample with ~~a~~ an amplification reagent mixture, in either case, the amplification reagent mixture being free of bumper primers and comprising:

(i) ~~the genomic DNA comprising the target polynucleotide sequence,~~

~~(ii)~~ a polymerase,

~~(iii)~~ (ii) a nicking enzyme,

~~(iv)~~ (iii) a first oligonucleotide comprising a nicking site and a nicking enzyme binding site, ~~and~~

~~(v)~~ (iv) a second oligonucleotide comprising a nicking site and a nicking enzyme binding site, and

~~b)~~ (c) subjecting the reaction mixture formed by the step of combining to essentially isothermal conditions to amplify the target polynucleotide sequence without the assistance of bumper primers, and

(d) detecting the amplified target polynucleotide sequence in real time within 10 minutes of subjecting the reaction mixture to essentially isothermal conditions.

96. (Previously Presented) The method of claim 67, wherein the target polynucleotide sequence is amplified about 1E+8-fold.

97. (Previously Presented) The method of claim 67, wherein the target polynucleotide sequence is amplified about 3E+9-fold.

98. (Previously Presented) The method of claim 67, wherein the target polynucleotide sequence is amplified about 7E+10-fold.

99. (Previously Presented) The method of claim 67, wherein amplification of the target polynucleotide sequence is performed at a constant temperature for about 1 to 12 minutes.

100. (Previously Presented) The method of claim 67, wherein amplification of the target polynucleotide sequence is performed at a constant temperature for about 1 to 10 minutes.

101. (Previously Presented) The method of claim 67, wherein amplification of the target polynucleotide sequence is performed at a constant temperature for about 1 to 8 minutes.

102. (Previously Presented) The method of claim 67, wherein amplification of the target polynucleotide sequence is performed at a constant temperature for about 1 to 5 minutes.

103. (Previously Presented) The method of claim 67, wherein amplification of the target polynucleotide sequence is performed at a constant temperature for about 1 to 2.5 minutes.

104. (Previously Presented) The method of claim 67, wherein amplification of the target polynucleotide sequence is performed at a constant temperature for about 2.5 to 5 minutes.

105. (Previously Presented) The method of claim 67, wherein amplification of the target polynucleotide sequence is performed at a constant temperature for about 2.5 to 8 minutes.

106. (Previously Presented) The method of claim 67, wherein amplification of the target polynucleotide sequence is performed at a constant temperature for about 2.5 to 10 minutes.

107. (Currently Amended) A method of amplifying a target polynucleotide sequence ~~of a target nucleic acid present in a sample obtained from an animal~~, the method comprising:

(a) obtaining, from an animal, plant or food, a sample comprising a target nucleic acid, the target nucleic acid comprising the target polynucleotide sequence,

~~(a)~~ (b) ~~preparing,~~ without first subjecting the target nucleic acid to a thermal denaturation step associated with amplification of the target polynucleotide sequence, combining, in a single step, the obtained sample directly with an amplification reagent mixture or diluting the obtained sample and combining, in a single step, the diluted sample with ~~a~~ an amplification reagent mixture, in either case, the amplification reagent mixture being free of bumper primers and comprising:

(i) ~~the target nucleic acid comprising the target polynucleotide sequence,~~

~~(ii)~~ a polymerase,

~~(iii)~~ (ii) a nicking enzyme,

~~(iv)~~ (iii) a first oligonucleotide comprising a nicking site and a nicking enzyme binding site, and

~~(v)~~ (iv) a second oligonucleotide comprising a nicking site and a nicking enzyme binding site, ~~and~~

~~(b)~~ (c) subjecting the reaction mixture formed by the step of combining to essentially isothermal conditions to amplify the target polynucleotide sequence without the assistance of bumper primers,

(d) detecting the amplified target polynucleotide sequence in real time within 10 minutes of subjecting the reaction mixture to essentially isothermal conditions, and

wherein the target polynucleotide sequence is amplified about 1E+8-fold in less than about ~~1 to~~

Attorney Docket No.: 30171-0025003 / Client Ref: ITI-001

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

First Named Inventor	:	Brian K. Maples	Art Unit	:	1637
Serial No.	:	14/067,623	Examiner	:	Angela Marie Bertagna
Filed	:	October 30, 2013	Conf. No.	:	8019
Title	:	NICKING AND EXTENSION AMPLIFICATION REACTION FOR THE EXPONENTIAL AMPLIFICATION OF NUCLEIC ACIDS			

ATTN: Examiner Bertanga

RE: Proposed Amendment following Examienr Interview of August 26, 2016

FAX: 571-273-8291

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List of claims.

1. -66. (Canceled)

67. (Currently Amended) A method of amplifying a target polynucleotide sequence of a target nucleic acid, the method comprising:

(a) obtaining, from an animal, a sample comprising a target nucleic acid, the target nucleic acid comprising the target polynucleotide sequence.

(b) preparing, without first subjecting the target nucleic acid to a thermal denaturation step associated with amplification of the target polynucleotide sequence, combining, in a single step, the obtained sample directly with an amplification reagent mixture or diluting the obtained sample and combining, in a single step, the diluted sample with a an amplification reagent mixture, in either case, the amplification reagent mixture being free of bumper primers and comprising:

(i) the target nucleic acid comprising the target polynucleotide sequence,

(ii) a polymerase,

(iii) (ii) a nicking enzyme,

(iv) (iii) a first oligonucleotide comprising a nicking site and a nicking enzyme binding site, and

(v) (iv) a second oligonucleotide comprising a nicking site and a nicking enzyme binding site; and

(c) amplifying the target polynucleotide sequence, without the assistance of bumper primers, under by subjecting the reaction mixture formed by the step of combining to essentially isothermal conditions, from steps comprising:

(a) (i) forming a first duplex comprising the target polynucleotide sequence and the first oligonucleotide;

(b) (ii) extending, using the polymerase, the first oligonucleotide along the target polynucleotide sequence to form an extended first oligonucleotide comprising a sequence complementary to the second oligonucleotide;

~~(c)~~ (iii) forming a second duplex comprising the second oligonucleotide and the extended first oligonucleotide;

~~(d)~~ (iv) extending, using the polymerase, the second oligonucleotide along the extended first oligonucleotide to form a third duplex comprising an extended second oligonucleotide comprising a sequence complementary to the first oligonucleotide and a first double-stranded nicking enzyme binding site;

~~(e)~~ (v) nicking, with the nicking enzyme, the first nicking site on the third duplex to produce a fourth duplex comprising the extended second oligonucleotide and a fragment of the extended first oligonucleotide; and

~~(f)~~ (vi) extending, using the polymerase, the fragment of the extended first oligonucleotide along the extended second oligonucleotide of the fourth duplex to produce a double-stranded nucleic acid product ~~and~~ containing a second double-stranded nicking enzyme binding site, and

(d) detecting the amplified target polynucleotide sequence in real time within 10 minutes of subjecting the reaction mixture to essentially isothermal conditions ~~wherein amplification of the target polynucleotide sequence is performed at a constant temperature for about 1 to 12 minutes~~.

68. (Currently Amended) The method of claim 67, wherein the target nucleic acid is obtained from an animal pathogen and the double-stranded nucleic acid product comprises:

i) a first strand and a second strand, wherein the first strand comprises a first polynucleotide sequence corresponding to the target polynucleotide sequence and the second strand comprises a second polynucleotide sequence complementary to the target polynucleotide sequence, and

ii) first and second double-stranded nicking sites spaced apart by the target polynucleotide sequence.

69. (Currently Amended) The method of claim 67, wherein the target nucleic acid is obtained from an animal pathogen and the target polynucleotide sequence is amplified from steps further comprising:

~~a)~~ vii) nicking, using the nicking enzyme, the first nicking site of the double-stranded nucleic acid product to produce a fifth duplex comprising a first polynucleotide sequence corresponding to the target polynucleotide sequence and a fragment of the first oligonucleotide, and nicking, using the nicking enzyme, the second nicking site of the double-stranded nucleic acid product to produce a sixth duplex comprising a second polynucleotide sequence complementary to the target polynucleotide sequence and a fragment of the second oligonucleotide;

~~b)~~ viii) extending, using the polymerase, the fragment of the first oligonucleotide along the first polynucleotide sequence of the fifth duplex to produce a first double stranded product comprising a copy of the nicking site and a copy of the first polynucleotide sequence and extending, using the polymerase, the fragment of the second oligonucleotide along the second polynucleotide sequence of the sixth duplex to produce a second double stranded product comprising a copy of the nicking site and a copy of the second polynucleotide sequence; and

~~c)~~ ix) nicking, using the nicking enzyme, the copy of the nicking site of the first double stranded product to release a copy of the first polynucleotide sequence and nicking, using the nicking enzyme, the copy of the nicking site of the second double stranded product to release a copy of the second polynucleotide sequence.

70. (Withdrawn, Currently Amended) The method of claim ~~71~~ 67, wherein the animal is a human.

71.-74. (Cancelled)

75. (Canceled) ~~The method of claim 67, wherein the target nucleic acid is obtained from an animal pathogen.~~

76. (Currently Amended) The method of claim ~~75~~68, wherein the animal pathogen is a single-stranded DNA virus, double-stranded DNA virus, or single-stranded RNA virus.

77. (Withdrawn, Currently Amended) The method of claim ~~75~~68, wherein the animal pathogen is a bacterium.

78. (Withdrawn, Currently Amended) The method of claim ~~75~~68, wherein the animal pathogen contains spores and the target polynucleotide is amplified from the spores without the need for lysis of the spores.

79. (Currently Amended) The method of claim ~~74~~67, wherein the sample obtained from an animal is ~~obtained from the~~ blood, ~~bone marrow,~~ mucus, ~~lymph, liver, spleen, kidney, lungor ovary, biopsies,~~ sputum, saliva, tears, feces ~~faeces~~ or urine of the animal.

80. (Currently Amended) The method of claim 79, wherein the sample obtained from an animal is ~~obtained from the~~ mucus, sputum, or saliva of the animal.

81. (Withdrawn) The method of claim 67, wherein the target nucleic acid is double-stranded DNA.

82. (Withdrawn) The method of claim 67, wherein the target nucleic acid is single-stranded DNA.

83. (Previously Presented) The method of claim 67, wherein the target nucleic acid is RNA.

84. (Withdrawn) The method of claim 67, wherein the target nucleic acid is genomic DNA.

85. (Currently Amended) The method of claim 67, wherein the sample obtained from the animal is mucus, saliva or sputum and the target nucleic acid is viral DNA or viral RNA.

86. (Canceled)

87. (Previously Presented) The method of claim 67, wherein the nicking enzyme is Nt.BstNBI.

88. (Previously Presented) The method of claim 67, wherein the nicking enzyme does not nick within the target polynucleotide sequence.

89. (Canceled) ~~The method of claim 67, wherein amplification of the target polynucleotide sequence is performed without the use of temperature cycling.~~

90. (Previously Presented) The method of claim 67, wherein amplification of the target polynucleotide sequence is performed at about 55°C-59°C.

91. (Canceled)

92. (Currently Amended) The method of claim 67, ~~which~~ wherein the step of amplifying the target polynucleotide sequence is performed at a temperature higher than the melting temperature of the first oligonucleotide/target polynucleotide sequence complex.

93. (Cancelled)

94. (Currently Amended) The method of claim ~~93~~67, wherein the amplification product is detected by a detection method selected from the group consisting of ~~gel electrophoresis, mass spectrometry,~~ fluorescence, intercalating dye detection, fluorescence resonance energy transfer (FRET), molecular beacon detection, ~~surface capture, capillary electrophoresis,~~ and incorporation of labeled nucleotides ~~to allow detection by capture, fluorescence polarization, and lateral flow capture, or a combination thereof.~~

95. – 96. (Canceled)

97. (Previously Presented) The method of claim 67, wherein the target polynucleotide sequence is amplified about 1E+8-fold.

98. (Previously Presented) The method of claim 67, wherein the target polynucleotide sequence is amplified about 3E+9-fold.

99. (Previously Presented) The method of claim 67, wherein the target polynucleotide sequence is amplified about 7E+10-fold.

100. (Previously Presented) The method of claim 67, wherein amplification of the target polynucleotide sequence is performed at a constant temperature for about 1 to 10 minutes.

101. (Previously Presented) The method of claim 67, wherein amplification of the target polynucleotide sequence is performed at a constant temperature for about 1 to 8 minutes.

102. (Previously Presented) The method of claim 67, wherein amplification of the target polynucleotide sequence is performed at a constant temperature for about 1 to 5 minutes.

103. (Previously Presented) The method of claim 67, wherein amplification of the target polynucleotide sequence is performed at a constant temperature for about 1 to 2.5 minutes.

104. (Previously Presented) The method of claim 67, wherein amplification of the target polynucleotide sequence is performed at a constant temperature for about 2.5 to 5 minutes.

105. (Previously Presented) The method of claim 67, wherein amplification of the target polynucleotide sequence is performed at a constant temperature for about 2.5 to 8 minutes.

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106. (Previously Presented) The method of claim 67, wherein amplification of the target polynucleotide sequence is performed at a constant temperature for about 2.5 to 10 minutes.

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EAST Search History

EAST Search History (Prior Art)

Ref #	Hits	Search Query	DBs	Default Operator	Plurals	Time Stamp
S83	3	"6403341".pn. "6566103".pn. us-20080038782-$.did.	US-PGPUB; USPAT	OR	OFF	2016/08/24 15:31
S82	2	"6403341".pn. "6566103".pn. us-2008038782-$.did.	US-PGPUB; USPAT	OR	OFF	2016/08/24 15:31
S84	8	us-20040038256-$.did. us-20040058349-$.did. "5648211".pn. "6686150.pn"	US-PGPUB; USPAT; FPRS; EPO; JPO; DERWENT	OR	OFF	2016/08/26 13:06
S86	7569	blackburn.in.	US-PGPUB; USPAT; FPRS; EPO; JPO; DERWENT	OR	OFF	2016/08/26 13:18
S85	0	"6686150.pn"	US-PGPUB; USPAT; FPRS; EPO; JPO; DERWENT	OR	OFF	2016/08/26 13:18
S87	2	"5648211".pn. "6686150".pn.	USPAT	OR	OFF	2016/09/08 13:45
S88	2	us-20040038256-$.did. us-20040058349-$.did.	US-PGPUB	OR	OFF	2016/09/08 13:47
S89	1	us-20080038782-$.did.	US-PGPUB	OR	OFF	2016/09/08 14:24
S90	2	"6403341".pn. "6566103".pn.	USPAT	OR	OFF	2016/09/08 14:26
L6	5	(richard near2 roth).in. and (nicking or nick or nicked or nicks or nickase).clm.	US-PGPUB; USPAT; FPRS; EPO; JPO; DERWENT	OR	OFF	2016/09/08 15:42
L5	24	(Provins near2 Jarrod).in.	US-PGPUB; USPAT; FPRS; EPO; JPO; DERWENT	OR	OFF	2016/09/08 15:42
L4	4	(andrew near2 miller).in. and (nicking or nick or nicked or nicks or nickase).clm.	US-PGPUB; USPAT; FPRS; EPO; JPO; DERWENT	OR	OFF	2016/09/08 15:42
L3	34	(rebecca near2 holmberg).in.	US-PGPUB; USPAT; FPRS; EPO; JPO; DERWENT	OR	OFF	2016/09/08 15:42
L2	18	(brian near2 maples).in.	US-PGPUB; USPAT; FPRS; EPO; JPO; DERWENT	OR	OFF	2016/09/08 15:42
L1	9	(ionian).as. and (nicking or nick or nicked or nickase)	US-PGPUB; USPAT; FPRS; EPO; JPO; DERWENT	OR	OFF	2016/09/08 15:42

L7	38	(jeffrey near2 mandell).in.	US-PGPUB; USPAT; FPRS; EPO; JPO; DERWENT	OR	OFF	2016/09/08 15:43
L8	6	l1 not l2	US-PGPUB; USPAT; FPRS; EPO; JPO; DERWENT	OR	OFF	2016/09/08 15:46
L9	15	l2 not l1	US-PGPUB; USPAT; FPRS; EPO; JPO; DERWENT	OR	OFF	2016/09/08 15:47
L10	16	l3 not l2 not l1	US-PGPUB; USPAT; FPRS; EPO; JPO; DERWENT	OR	OFF	2016/09/08 15:48
L14	20	l7 not l5 not l3 not l2 not l1	US-PGPUB; USPAT; FPRS; EPO; JPO; DERWENT	OR	OFF	2016/09/08 15:49
L13	0	l6 not l5 not l3 not l2 not l1	US-PGPUB; USPAT; FPRS; EPO; JPO; DERWENT	OR	OFF	2016/09/08 15:49
L12	6	l5 not l3 not l2 not l1	US-PGPUB; USPAT; FPRS; EPO; JPO; DERWENT	OR	OFF	2016/09/08 15:49
L11	0	l4 not l3 not l2 not l1	US-PGPUB; USPAT; FPRS; EPO; JPO; DERWENT	OR	OFF	2016/09/08 15:49
L32	1	((strand adj1 displac$) or sda) near8 ("1" adj1 (min or minutes))	US-PGPUB; USPAT; FPRS; EPO; JPO; DERWENT	OR	OFF	2016/09/08 15:54
L31	4	((strand adj1 displac$) or sda) near8 ("2" adj1 (min or minutes))	US-PGPUB; USPAT; FPRS; EPO; JPO; DERWENT	OR	OFF	2016/09/08 15:54
L30	24	((strand adj1 displac$) or sda) near8 ("3" adj1 (min or minutes))	US-PGPUB; USPAT; FPRS; EPO; JPO; DERWENT	OR	OFF	2016/09/08 15:54
L29	0	((strand adj1 displac$) or sda) near8 ("4" adj1 (min or minutes))	US-PGPUB; USPAT; FPRS; EPO; JPO; DERWENT	OR	OFF	2016/09/08 15:54
L28	67	((strand adj1 displac$) or sda) near8 ("5" adj1 (min or minutes))	US-PGPUB; USPAT; FPRS; EPO; JPO; DERWENT	OR	OFF	2016/09/08 15:54
L27	1	((strand adj1 displac$) or sda) near8 ("6" adj1 (min or minutes))	US-PGPUB; USPAT; FPRS; EPO; JPO; DERWENT	OR	OFF	2016/09/08 15:54
L26	0	((strand adj1 displac$) or sda) near8 ("7" adj1 (min or minutes))	US-PGPUB; USPAT; FPRS;	OR	OFF	2016/09/08 15:54

			EPO; JPO; DERWENT			
L25	0	((strand adj1 displac$) or sda) near8 ("8" adj1 (min or minutes))	US-PGPUB; USPAT; FPRS; EPO; JPO; DERWENT	OR	OFF	2016/09/08 15:54
L24	0	((strand adj1 displac$) or sda) near8 ("9" adj1 (min or minutes))	US-PGPUB; USPAT; FPRS; EPO; JPO; DERWENT	OR	OFF	2016/09/08 15:54
L23	41	((strand adj1 displac$) or sda) near8 ("10" adj1 (min or minutes))	US-PGPUB; USPAT; FPRS; EPO; JPO; DERWENT	OR	OFF	2016/09/08 15:54
L22	39	((strand adj1 displac$) or sda) near8 ("15" adj1 (min or minutes))	US-PGPUB; USPAT; FPRS; EPO; JPO; DERWENT	OR	OFF	2016/09/08 15:54
L34	16	((strand adj1 displac$) or sda) near8 (("without" or absence or lack$ or eliminat$ or omit$ or omission) near3 (bumper))	US-PGPUB; USPAT; FPRS; EPO; JPO; DERWENT	OR	ON	2016/09/08 15:55
L33	16	((strand adj1 displac$) or sda) near8 (("without" or absence) near3 (bumper))	US-PGPUB; USPAT; FPRS; EPO; JPO; DERWENT	OR	ON	2016/09/08 15:55
L35	37	l23 not l22	US-PGPUB; USPAT; FPRS; EPO; JPO; DERWENT	OR	OFF	2016/09/08 15:59
L36	52	l28 not l23 not l22	US-PGPUB; USPAT; FPRS; EPO; JPO; DERWENT	OR	OFF	2016/09/08 16:12
L37	23	l30 not l28 not l23 not l22	US-PGPUB; USPAT; FPRS; EPO; JPO; DERWENT	OR	OFF	2016/09/08 16:17
L38	0	l34 not l33	US-PGPUB; USPAT; FPRS; EPO; JPO; DERWENT	OR	ON	2016/09/08 16:27

EAST Search History (Interference)

Ref #	Hits	Search Query	DBs	Default Operator	Plurals	Time Stamp
L21	9	(jeffrey near2 mandell).in.	US-PGPUB; USPAT	OR	OFF	2016/09/08 15:50
L20	5	(richard near2 roth).in. and (nicking or nick or nicked or nicks or nickase).clm.	US-PGPUB; USPAT	OR	OFF	2016/09/08 15:50
L19	5	(Provins near2 Jarrod).in.	US-PGPUB; USPAT	OR	OFF	2016/09/08 15:50
L18	4	(andrew near2 miller).in. and (nicking or nick or nicked or nicks or nickase).clm.	US-PGPUB;	OR	OFF	2016/09/08 15:50

			USPAT			
L17	9	(rebecca near2 holmberg).in.	US-PGPUB; USPAT	OR	OFF	2016/09/08 15:50
L16	4	(brian near2 maples).in.	US-PGPUB; USPAT	OR	OFF	2016/09/08 15:50
L15	3	(ionian).as. and (nicking or nick or nicked or nickase)	US-PGPUB; USPAT	OR	OFF	2016/09/08 15:50
L40	40	((strand adj1 displac$) or sda) near8 ("10" adj1 (min or minutes))	US-PGPUB; USPAT	OR	OFF	2016/09/08 16:28
L39	39	((strand adj1 displac$) or sda) near8 ("15" adj1 (min or minutes))	US-PGPUB; USPAT	OR	OFF	2016/09/08 16:28
L46	0	((strand adj1 displac$) or sda) near8 ("4" adj1 (min or minutes))	US-PGPUB; USPAT	OR	OFF	2016/09/08 16:29
L45	60	((strand adj1 displac$) or sda) near8 ("5" adj1 (min or minutes))	US-PGPUB; USPAT	OR	OFF	2016/09/08 16:29
L44	1	((strand adj1 displac$) or sda) near8 ("6" adj1 (min or minutes))	US-PGPUB; USPAT	OR	OFF	2016/09/08 16:29
L43	0	((strand adj1 displac$) or sda) near8 ("7" adj1 (min or minutes))	US-PGPUB; USPAT	OR	OFF	2016/09/08 16:29
L42	0	((strand adj1 displac$) or sda) near8 ("8" adj1 (min or minutes))	US-PGPUB; USPAT	OR	OFF	2016/09/08 16:29
L41	0	((strand adj1 displac$) or sda) near8 ("9" adj1 (min or minutes))	US-PGPUB; USPAT	OR	OFF	2016/09/08 16:29
L50	16	((strand adj1 displac$) or sda) near8 (("without" or absence) near3 (bumper))	US-PGPUB; USPAT	OR	ON	2016/09/08 16:30
L49	1	((strand adj1 displac$) or sda) near8 ("1" adj1 (min or minutes))	US-PGPUB; USPAT	OR	OFF	2016/09/08 16:30
L48	4	((strand adj1 displac$) or sda) near8 ("2" adj1 (min or minutes))	US-PGPUB; USPAT	OR	OFF	2016/09/08 16:30
L47	22	((strand adj1 displac$) or sda) near8 ("3" adj1 (min or minutes))	US-PGPUB; USPAT	OR	OFF	2016/09/08 16:30

9/8/2016 4:32:44 PM
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Substitute Disclosure Form U.S. Department of Commerce Patent and Trademark Office	Attorney Docket No. 30171-0025002	Application No. 14/067,620
Information Disclosure Statement by Applicant (Use several sheets if necessary)	Applicant Ionian Technologies Inc.	
(37 CFR §1.98(b))	Filing Date October 30, 2013	Group Art Unit 1637

U.S. Patent Documents							
Examiner Initial	Desig. ID	Document Number	Publication Date	Patentee	Class	Subclass	Filing Date If Appropriate
/A.B./	1.	6,403,341	06/11/2002	Barnes et al.			
/A.B./	2.	6,566,103	05/20/2003	Wijnhoven et al.			
/A.B./	3.	2008/038782	02/14/2008	Borns			

Foreign Patent Documents or Published Foreign Patent Applications								
Examiner Initial	Desig. ID	Document Number	Publication Date	Country or Patent Office	Class	Subclass	Translation Yes	Translation No
/A.B./	4.	WO2015/113828	08/06/2015	WIPO				
/A.B./	5.	WO2016/004333	01/07/2016	WIPO				
/A.B./	6.	WO2003/012066	02/13/2003	WIPO				
/A.B./	7.	WO2007/096182	08/30/2007	WIPO				
/A.B./	8.	GB 2416352	01/25/2006	United Kingdom				
/A.B./	9.	WO2011/085160	07/14/2011	WIPO				
/A.B./	10.	EP 2824189	01/14/2015	Europe				

Other Documents (include Author, Title, Date, and Place of Publication)		
Examiner Initial	Desig. ID	Document
/A.B./	11.	Extended European Search Report in corresponding Application No. 13799829.0, dated March 31, 2016, pages 1-10
/A.B./	12.	Arena et al., "Calcium- And Magnesium-EDTA Complexes. Stability Constants and Their Dependence on Temperature and Ionic Strength," Thermochimica Acta, 61 (1983) 129-138
/A.B./	13.	Examiner's Report in corresponding Canadian Application No. 2,693,805 dated March 18, 2016, pages 1-5

Examiner Signature /Angela Bertagna/	Date Considered 09/07/2016
EXAMINER: Initials citation considered. Draw line through citation if not in conformance and not considered. Include copy of this form with next communication to applicant.	

Substitute Disclosure Form

Substitute Disclosure Form U.S. Department of Commerce Patent and Trademark Office	Attorney Docket No. 30171-0025002	Application No. 14/067,620
Information Disclosure Statement by Applicant (Use several sheets if necessary)	Applicant Ionian Technologies Inc.	
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U.S. Patent Documents							
Examiner Initial	Desig. ID	Document Number	Publication Date	Patentee	Class	Subclass	Filing Date If Appropriate
/A.B./	1.	2004/0038256	2/26/2004	Van Ness et al.			
/A.B./	2.	2004/0058349	3/25/2004	Van Ness et al.			
/A.B./	3.	5,648,211	7/15/1997	Fraiser et al.			
/A.B./	4.	6,686,150	2/3/2004	Blackburn et al.			

Foreign Patent Documents or Published Foreign Patent Applications								
Examiner Initial	Desig. ID	Document Number	Publication Date	Country or Patent Office	Class	Subclass	Translation Yes	Translation No
	5.							

Other Documents (include Author, Title, Date, and Place of Publication)		
Examiner Initial	Desig. ID	Document
/A.B./	6.	Little et al., "Molecular Diagnostics and Genetics," Clinical Chemistry, 45:6, 777-784 (1999)
/A.B./	7.	Wang et al., "Homogeneous Real-Time Detection of Single-Nucleotide Polymorphisms by Strand Displacement Amplification on the BD ProbeTec ET System," Clinical Chemistry, 49:10, 1599-1607 (2003)
/A.B./	8.	C.A. Spargo et al., "Detection of M. tuberculosis DNA using Thermophilic Strand Displacement Amplification," Molecular and Cellular Probes (1996) 10, 247-256
/A.B./	9.	Chinese Office Action in Application No. 201410465144.4, dated June 28, 2016, pages 1-9
/A.B./	10.	Notification of Reexamination in Chinese Application No. 200880105424.7, pages 1-8 (2016)
/A.B./	11.	Chinese Office Action in Application No. 201410466581.8, dated January 21, 2016, pages 1-20
/A.B./	12.	Chinese Office Action in Application No. 201410465144.4, dated December 1, 2015, pages 1-7
/A.B./	13.	Ehses et al., "Optimization and design of oligonucleotide setup for strand displacement amplification," J. Biochem. Biophys. Methods, 63 (2005) 170-186
/A.B./	14.	Artificial DNA: Methods and Applications (2003), Khudyakov Y.E. & Fields, H.A. (Eds), *Synthetic DNA Used in Amplification Reactions* (pp. 115-159), CRC Press LLC
/A.B./	15.	Nadezhda V. Zyrina et al., "N.BspD61 DNA nickase strongly stimulates template-independent synthesis of non-palindromic repetitive DNA by Bst DNA polymerase," Biol. Chem. Vol. 388, pp. 367-372, April 2007

Examiner Signature /Angela Bertagna/	Date Considered 09/07/2016
EXAMINER: Initials citation considered. Draw line through citation if not in conformance and not considered. Include copy of this form with next communication to applicant.	

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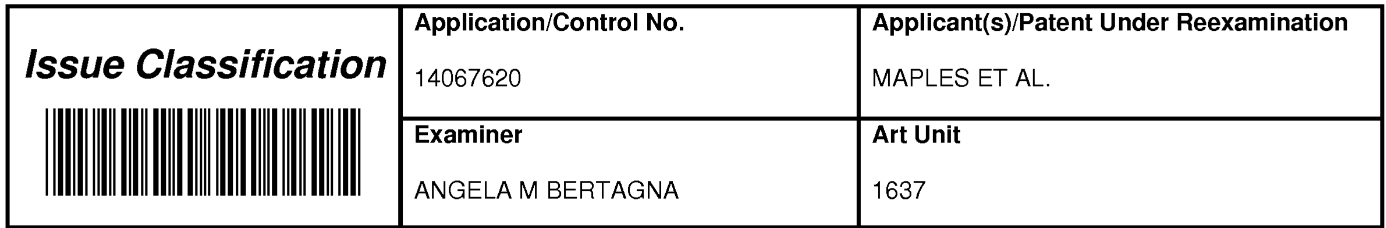

Issue Classification	Application/Control No.	Applicant(s)/Patent Under Reexamination
	14067620	MAPLES ET AL.
	Examiner	**Art Unit**
	ANGELA M BERTAGNA	1637

CPC

Symbol				Type	Version
C12Q	1	/	686	F	2013-01-01
C12Q	1	/	6844	I	2013-01-01
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CPC Combination Sets

Symbol				Type	Set	Ranking	Version
C12Q	1	/	6844	I	1	1	2013-01-01
C12Q	2527	/	101	A	1	2	2013-01-01
C12Q	2525	/	161	A	1	3	2013-01-01
C12Q	2525	/	131	A	1	4	2013-01-01
C12Q	1	/	6844	I	2	1	2013-01-01
C12Q	2527	/	101	A	2	2	2013-01-01
C12Q	2525	/	131	A	2	3	2013-01-01
C12Q	2537	/	137	A	2	4	2013-01-01

		Total Claims Allowed:	
NONE (Assistant Examiner)	(Date)	35	
/ANGELA M BERTAGNA/ Primary Examiner.Art Unit 1637 (Primary Examiner)	9/8/2016 (Date)	O.G. Print Claim(s) 1	O.G. Print Figure NONE

U.S. Patent and Trademark Office — Part of Paper No. 20160826

Issue Classification	Application/Control No. 14067620	Applicant(s)/Patent Under Reexamination MAPLES ET AL.
	Examiner ANGELA M BERTAGNA	Art Unit 1637

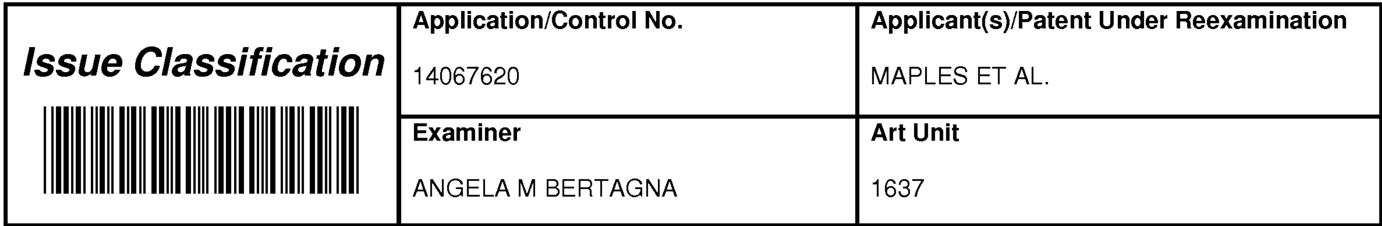
US ORIGINAL CLASSIFICATION					
CLASS		SUBCLASS			
CROSS REFERENCE(S)					
CLASS	SUBCLASS (ONE SUBCLASS PER BLOCK)				

INTERNATIONAL CLASSIFICATION									
CLAIMED					NON-CLAIMED				
C	1	2	Q	1 / 68 (2006.01.01)					

NONE (Assistant Examiner)	(Date)	Total Claims Allowed: 35	
/ANGELA M BERTAGNA/ Primary Examiner.Art Unit 1637 (Primary Examiner)	9/8/2016 (Date)	O.G. Print Claim(s) 1	O.G. Print Figure NONE

U.S. Patent and Trademark Office

Part of Paper No. 20160826



Issue Classification	Application/Control No.	Applicant(s)/Patent Under Reexamination
	14067620	MAPLES ET AL.
	Examiner	**Art Unit**
	ANGELA M BERTAGNA	1637

☐ **Claims renumbered in the same order as presented by applicant** ☐ **CPA** ☒ **T.D.** ☐ **R.1.47**

Final	Original	Final	Original	Final	Original	Final	Original	Final	Original	Final	Original	Final	Original	Final	Original
	1		17		33		49		65	15	81	25	97		
	2		18		34		50		66	16	82	26	98		
	3		19		35		51	1	67	17	83	27	99		
	4		20		36		52	2	68		84	28	100		
	5		21		37		53	3	69	18	85	29	101		
	6		22		38		54	4	70	19	86	30	102		
	7		23		39		55	5	71		87	31	103		
	8		24		40		56	6	72	20	88	32	104		
	9		25		41		57	7	73		89	33	105		
	10		26		42		58	8	74	21	90	34	106		
	11		27		43		59	9	75		91	35	107		
	12		28		44		60	10	76	22	92				
	13		29		45		61	11	77		93				
	14		30		46		62	12	78		94				
	15		31		47		63	13	79	23	95				
	16		32		48		64	14	80	24	96				

NONE (Assistant Examiner)	(Date)	**Total Claims Allowed:** 35	
/ANGELA M BERTAGNA/ Primary Examiner.Art Unit 1637 (Primary Examiner)	9/8/2016 (Date)	O.G. Print Claim(s) 1	O.G. Print Figure NONE

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BIB DATA SHEET

CONFIRMATION NO. 4288

SERIAL NUMBER	FILING or 371(c) DATE	CLASS	GROUP ART UNIT	ATTORNEY DOCKET NO.
14/067,620	10/30/2013 **RULE**	435	1637	30171-0025002 / ITI-001

APPLICANTS
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Jarrod Provins, Dana Point, CA;
Richard Roth, Carlsbad, CA;
Jeffrey Mandell, San Diego, CA;

** **CONTINUING DATA** *************************
This application is a CON of 11/778,018 07/14/2007

** **FOREIGN APPLICATIONS** *************************

** **IF REQUIRED, FOREIGN FILING LICENSE GRANTED** **
11/21/2013

Foreign Priority claimed ❑ Yes ☑ No 35 USC 119(a-d) conditions met ❑ Yes ☑ No Verified and Acknowledged /ANGELA MARIE BERTAGNA/ Examiner's Signature	❑ Met after Allowance Initials	STATE OR COUNTRY	SHEETS DRAWINGS	TOTAL CLAIMS	INDEPENDENT CLAIMS
		CA	24	29	3

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UNITED STATES

TITLE
Nicking and Extension Amplification Reaction for the Exponential Amplification of Nucleic Acids

FILING FEE RECEIVED	FEES: Authority has been given in Paper	
2620	FEES: Authority has been given in Paper No.____________ to charge/credit DEPOSIT ACCOUNT No.____________ for following:	❑ All Fees ❑ 1.16 Fees (Filing) ❑ 1.17 Fees (Processing Ext. of time) ❑ 1.18 Fees (Issue) ❑ Other ________________ ❑ Credit

BIB (Rev. 05/07).

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

First Named Inventor	:	Brian K. Maples	Art Unit	:	1637
Serial No.	:	14/067,620	Examiner	:	Angela Marie Bertagna
Filed	:	October 30, 2013	Conf. No.	:	4288
Title	:	NICKING AND EXTENSION AMPLIFICATION REACTION FOR THE EXPONENTIAL AMPLIFICATION OF NUCLEIC ACIDS			

Commissioner for Patents
P.O. Box 1450
Alexandria, VA 22313-1450

SUPPLEMENTAL AMENDMENT

Please amend the application as indicated on the following pages.

Amendments to the Claims:

This listing of claims replaces all prior versions and listings of claims in the application:

Listing of Claims:

1. - 66. (Canceled)

67. (Currently Amended) A method of amplifying a target polynucleotide sequence ~~of a target nucleic acid present in a sample obtained from an animal~~, the method comprising:
(a) obtaining, from an animal, plant or food, a sample comprising a target nucleic acid, the target nucleic acid comprising the target polynucleotide sequence,

~~(a)~~ (b) ~~preparing,~~ without first subjecting the target nucleic acid to a thermal denaturation step associated with amplification of the target polynucleotide sequence, combining, in a single step, the obtained sample directly with an amplification reagent mixture or diluting the obtained sample and combining, in a single step, the diluted sample with ~~a~~ an amplification reagent mixture, in either case, the amplification reagent mixture being free of bumper primers and comprising:

(i) ~~the target nucleic acid comprising the target polynucleotide sequence,~~
~~(ii)~~ a polymerase,
~~(iii)~~ (ii) a nicking enzyme,
~~(iv)~~ (iii) a first oligonucleotide comprising a nicking site and a nicking enzyme binding site, and
~~(v)~~ (iv) a second oligonucleotide comprising a nicking site and a nicking enzyme binding site, ~~and~~

~~(b)~~ (c) subjecting the reaction mixture formed by the step of combining to essentially isothermal conditions to amplify the target polynucleotide sequence without the assistance of bumper primers, and

(d) detecting the amplified target polynucleotide sequence in real time within 10 minutes of subjecting the reaction mixture to essentially isothermal conditions.

68. (Currently Amended) The method of claim 67, wherein the sample is obtained from an animal and the target polynucleotide sequence is amplified from steps comprising:

(a) forming a first duplex comprising the target polynucleotide sequence and the first oligonucleotide;

(b) extending, using the polymerase, the first oligonucleotide along the target polynucleotide sequence to form an extended first oligonucleotide comprising a sequence complementary to the second oligonucleotide;

(c) forming a second duplex comprising the second oligonucleotide and the extended first oligonucleotide;

(d) extending, using the polymerase, the second oligonucleotide along the extended first oligonucleotide to form a third duplex comprising an extended second oligonucleotide comprising a sequence complementary to the first oligonucleotide and a first double-stranded nicking enzyme binding site;

(e) nicking, with the nicking enzyme, the first nicking site on the third duplex to produce a fourth duplex comprising the extended second oligonucleotide and a fragment of the extended first oligonucleotide; and

(f) extending, using the polymerase, the fragment of the extended first oligonucleotide along the extended second oligonucleotide of the fourth duplex to produce a double-stranded nucleic acid product ~~and~~ containing a second double-stranded nicking enzyme binding site.

69. (Currently Amended) The method of claim 68, wherein sample is obtained from an animal and the double-stranded nucleic acid product comprises:

i) a first strand and a second strand, wherein the first strand comprises a first polynucleotide sequence corresponding to the target polynucleotide sequence and the second strand comprises a second polynucleotide sequence complementary to the target polynucleotide sequence, and

ii) first and second double-stranded nicking sites spaced apart by the target polynucleotide sequence.

70. (Currently Amended) The method of claim 68, further comprising the steps of:

~~a)~~ g) nicking, using the nicking enzyme, the first nicking site of the double-stranded nucleic acid product to produce a fifth duplex comprising a first polynucleotide sequence corresponding to the target polynucleotide sequence and a fragment of the first oligonucleotide, and nicking, using the nicking enzyme, the second nicking site of the double-stranded nucleic acid product to produce a sixth duplex comprising a second polynucleotide sequence complementary to the target polynucleotide sequence and a fragment of the second oligonucleotide;

~~b)~~ h) extending, using the polymerase, the fragment of the first oligonucleotide along the first polynucleotide sequence of the fifth duplex to produce a first double stranded product comprising a copy of the nicking site and a copy of the first polynucleotide sequence and extending, using the polymerase, the fragment of the second oligonucleotide along the second polynucleotide sequence of the sixth duplex to produce a second double stranded product comprising a copy of the nicking site and a copy of the second polynucleotide sequence; and

~~c)~~ i) nicking, using the nicking enzyme, the copy of the nicking site of the first double stranded product to release a copy of the first polynucleotide sequence and nicking, using the nicking enzyme, the copy of the nicking site of the second double stranded product to release a copy of the second polynucleotide sequence.

71. (Currently Amended) The method of claim 67, wherein the sample is obtained from an animal and the animal is a human, the target nucleic acid is a target nucleic acid of a human pathogen, and the sample is obtained from the mucus, sputum, or saliva of the human.

72. (Currently Amended) The method of claim 67, wherein the sample is obtained from a human and the target nucleic acid is ~~obtained from~~ a target nucleic acid of a human ~~an animal~~ pathogen.

73. (Currently Amended) The method of claim 72, wherein the human ~~animal~~ pathogen is a single-stranded DNA virus, double-stranded DNA virus, or single-stranded RNA virus.

74. (Withdrawn, Currently Amended) The method of claim 72, wherein the human ~~animal~~ pathogen is a bacterium.

75. (Withdrawn, Currently Amended) The method of claim 72, wherein the human ~~animal~~ pathogen contains spores and the target polynucleotide is amplified from the spores without the need for lysis of the spores.

76. (Currently Amended) The method of claim 67, wherein the animal is human and the sample ~~obtained from an animal~~ is ~~obtained from the~~ blood, ~~bone marrow,~~ mucus, ~~lymph, hard tissues, biopsies,~~ sputum, saliva, tears, feces ~~faeces~~ or urine ~~of the animal~~.

77. (Currently Amended) The method of claim 76, wherein the sample ~~obtained from an animal~~ is ~~obtained from the~~ mucus, sputum, or saliva ~~of the animal~~.

78. (Withdrawn) The method of claim 67, wherein the target nucleic acid is double-stranded DNA.

79. (Withdrawn) The method of claim 67, wherein the target nucleic acid is single-stranded DNA.

80. (Previously Presented) The method of claim 67, wherein the target nucleic acid is RNA.

81. (Withdrawn) The method of claim 67, wherein the target nucleic acid is selected from the group consisting of genomic DNA, plasmid DNA, viral DNA, mitochondrial DNA, cDNA, synthetic double-stranded DNA and synthetic single-stranded DNA.

82. (Withdrawn) The method of claim 81, wherein the target nucleic acid is genomic DNA.

83. (Previously Presented) The method of claim 67, wherein the target nucleic acid is viral DNA or viral RNA.

84. (Canceled)

85. (Previously Presented) The method of claim 67, wherein the nicking enzyme is Nt.BstNBI.

86. (Previously Presented) The method of claim 67, wherein the nicking enzyme does not nick within the target polynucleotide sequence.

87. (Canceled)

88. (Previously Presented) The method of claim 67, wherein amplification of the target polynucleotide sequence is performed at about 55°C-59°C.

89. (Canceled)

90. (Currently Amended) The method of claim ~~68~~ 67, ~~which~~ wherein the step of amplifying the target polynucleotide sequence is performed at a temperature higher than the melting temperature of the first oligonucleotide/target polynucleotide sequence complex.

91. (Canceled)

92. (Currently Amended) The method of claim 67 ~~91~~, wherein the amplification product is detected by a detection method selected from the group consisting of ~~gel~~

~~electrophoresis, mass spectrometry~~, fluorescence, intercalating dye detection, fluorescence resonance energy transfer (FRET), molecular beacon detection, ~~surface capture,~~ ~~capillary electrophoresis,~~ and incorporation of labeled nucleotides~~ to allow detection by capture, fluorescence polarization, and lateral flow capture, or a combination thereof~~.

93. (Canceled)

94. (Canceled)

95. (Withdrawn, Currently Amended) A method of amplifying a target polynucleotide sequence~~ of genomic DNA present in a sample obtained from an animal~~, the method comprising:

(a) obtaining, from a human, plant, or food, a sample containing genomic DNA, the genomic DNA comprising a target nucleic acid, the target nucleic acid comprising the target polynucleotide sequence,

(b) ~~preparing,~~ without first subjecting the genomic DNA to a thermal denaturation step associated with amplification of the target polynucleotide sequence, combining, in a single step, the obtained sample directly with an amplification reagent mixture or diluting the obtained sample and combining, in a single step, the diluted sample with ~~a~~ an amplification reagent mixture, in either case, the amplification reagent mixture being free of bumper primers and comprising:

(i) ~~the genomic DNA comprising the target polynucleotide sequence,~~

~~(ii)~~ a polymerase,

~~(iii)~~ (ii) a nicking enzyme,

~~(iv)~~ (iii) a first oligonucleotide comprising a nicking site and a nicking enzyme binding site, ~~and~~

~~(v)~~ (iv) a second oligonucleotide comprising a nicking site and a nicking enzyme binding site, and

~~b)~~ (c) subjecting the reaction mixture formed by the step of combining to essentially isothermal conditions to amplify the target polynucleotide sequence without the assistance of bumper primers, and

(d) detecting the amplified target polynucleotide sequence in real time within 10 minutes of subjecting the reaction mixture to essentially isothermal conditions.

96. (Previously Presented) The method of claim 67, wherein the target polynucleotide sequence is amplified about 1E+8-fold.

97. (Previously Presented) The method of claim 67, wherein the target polynucleotide sequence is amplified about 3E+9-fold.

98. (Previously Presented) The method of claim 67, wherein the target polynucleotide sequence is amplified about 7E+10-fold.

99. (Previously Presented) The method of claim 67, wherein amplification of the target polynucleotide sequence is performed at a constant temperature for about 1 to 12 minutes.

100. (Previously Presented) The method of claim 67, wherein amplification of the target polynucleotide sequence is performed at a constant temperature for about 1 to 10 minutes.

101. (Previously Presented) The method of claim 67, wherein amplification of the target polynucleotide sequence is performed at a constant temperature for about 1 to 8 minutes.

102. (Previously Presented) The method of claim 67, wherein amplification of the target polynucleotide sequence is performed at a constant temperature for about 1 to 5 minutes.

103. (Previously Presented) The method of claim 67, wherein amplification of the target polynucleotide sequence is performed at a constant temperature for about 1 to 2.5 minutes.

104. (Previously Presented) The method of claim 67, wherein amplification of the target polynucleotide sequence is performed at a constant temperature for about 2.5 to 5 minutes.

105. (Previously Presented) The method of claim 67, wherein amplification of the target polynucleotide sequence is performed at a constant temperature for about 2.5 to 8 minutes.

106. (Previously Presented) The method of claim 67, wherein amplification of the target polynucleotide sequence is performed at a constant temperature for about 2.5 to 10 minutes.

107. (Currently Amended) A method of amplifying a target polynucleotide sequence~~ of a target nucleic acid present in a sample obtained from an animal~~, the method comprising:

(a) obtaining, from an animal, plant or food, a sample comprising a target nucleic acid, the target nucleic acid comprising the target polynucleotide sequence,

~~(a)~~ (b) ~~preparing,~~ without first subjecting the target nucleic acid to a thermal denaturation step associated with amplification of the target polynucleotide sequence, combining, in a single step, the obtained sample directly with an amplification reagent mixture or diluting the obtained sample and combining, in a single step, the diluted sample with ~~a~~ an amplification reagent mixture, in either case, the amplification reagent mixture being free of bumper primers and comprising:

(i) ~~the target nucleic acid comprising the target polynucleotide sequence,~~

~~(ii)~~ a polymerase,

~~(iii)~~ (ii) a nicking enzyme,

~~(iv)~~ (iii) a first oligonucleotide comprising a nicking site and a nicking enzyme binding site, and

~~(v)~~ (iv) a second oligonucleotide comprising a nicking site and a nicking enzyme binding site, ~~and~~

~~(b)~~ (c) subjecting the reaction mixture formed by the step of combining to essentially isothermal conditions to amplify the target polynucleotide sequence without the assistance of bumper primers,

(d) detecting the amplified target polynucleotide sequence in real time within 10 minutes of subjecting the reaction mixture to essentially isothermal conditions, and

wherein the target polynucleotide sequence is amplified about 1E+8-fold in <u>less than</u> about ~~1 to~~ 12 minutes.

REMARKS

Upon entry of the above amendment, claims 67-83, 85-86, 88, 90, 92 and 95-107 will be pending. Claims 74-75, 78-79, 81-82 and 95 were previously withdrawn, so are not currently under examination. Claims 1-67, 84, 89 and 93-94 were previously canceled. Claims 87 and 91 is newly canceled, and claims 67-77, 90, 92, 95 and 107 have been amended. Most of the amendments are simply to clarify scope and are supported throughout the specification so that no new matter is introduced. For example, the amendments to claims 67, 95 and 107 find support at page 18, lines 3-5 and 12-13; page 26, lines 15-17; page 36, lines 29-30; page 32, line 25; Figures 6-8, and the Examples.

Applicant believes that the amendments submitted by the Applicant in the AMENDMENT AND REPLY TO FINAL ACTION OF FEBRUARY 11, 2016 filed August 11, 2016 have not been entered and the amendments submitted herewith reflect this. Applicant requests that the Office consider the Remarks presented in the August 11, 2016 submission in light of the present amendment.

No new matter has been introduced by these amendments. Reconsideration and allowance of the claims are respectfully requested in view of the above amendments and the following remarks.

Interview Summary

Applicant thanks Examiner Bertagna for the courtesy of a telephonic interview with Applicant's representatives Ian Lodovice, Belinda Lew and Jay Fister on August 26, 2016. During the interview, the participants discussed amendments for placing claims in conditions for allowance. The amendments to claims presented above are substantially as discussed by the participants during the interview.

Double Patenting

Without conceding the propriety of the obviousness-type double patenting rejections, Applicant submits herewith terminal disclaimers obviating these rejections, so the rejections should be withdrawn

CONCLUSION

In light of the arguments made herein, Applicant submits that the pending claims are patentable and request early and favorable action thereon. If any issues remain, the Examiner is asked to telephone the undersigned at 617-956-5972 to arrange a time for an interview.

Applicant does not concede any positions of the Office that are not expressly addressed above, nor do applicants concede that there are not other good reasons for patentability of the presented claims or other claims.

Applicant asks that all claims be examined in view of the amendment to the claims.

While no fees are believed to be due, the office is authorized to apply any necessary charges or credits to Deposit Account 06-1050, referencing the above attorney docket number.

Respectfully submitted,

Date: September 2, 2016

/Ian J.S. Lodovice, Reg. No. 59,749/

Ian J. Lodovice
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23561606.doc

Electronic Acknowledgement Receipt

EFS ID:	26824800
Application Number:	14067620
International Application Number:	
Confirmation Number:	4288
Title of Invention:	Nicking and Extension Amplification Reaction for the Exponential Amplification of Nucleic Acids
First Named Inventor/Applicant Name:	Brian K. Maples
Customer Number:	26161
Filer:	Ian J.S. Lodovice/Mary Florczak
Filer Authorized By:	Ian J.S. Lodovice
Attorney Docket Number:	30171-0025002 / ITI-001
Receipt Date:	02-SEP-2016
Filing Date:	30-OCT-2013
Time Stamp:	12:51:30
Application Type:	Utility under 35 USC 111(a)

Payment information:

Submitted with Payment	no

File Listing:

Document Number	Document Description	File Name	File Size(Bytes)/ Message Digest	Multi Part /.zip	Pages (if appl.)
1		301710025002SuppAmendment.pdf	123959 fd87a8f2eeedbfc42d482d496727689a97cfdea3	yes	12

	Multipart Description/PDF files in .zip description		
	Document Description	**Start**	**End**
	Supplemental Response or Supplemental Amendment	1	1
	Claims	2	10
	Applicant Arguments/Remarks Made in an Amendment	11	12
Warnings:			
Information:			
	Total Files Size (in bytes):	123959	

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New Applications Under 35 U.S.C. 111
If a new application is being filed and the application includes the necessary components for a filing date (see 37 CFR 1.53(b)-(d) and MPEP 506), a Filing Receipt (37 CFR 1.54) will be issued in due course and the date shown on this Acknowledgement Receipt will establish the filing date of the application.

National Stage of an International Application under 35 U.S.C. 371
If a timely submission to enter the national stage of an international application is compliant with the conditions of 35 U.S.C. 371 and other applicable requirements a Form PCT/DO/EO/903 indicating acceptance of the application as a national stage submission under 35 U.S.C. 371 will be issued in addition to the Filing Receipt, in due course.

New International Application Filed with the USPTO as a Receiving Office
If a new international application is being filed and the international application includes the necessary components for an international filing date (see PCT Article 11 and MPEP 1810), a Notification of the International Application Number and of the International Filing Date (Form PCT/RO/105) will be issued in due course, subject to prescriptions concerning national security, and the date shown on this Acknowledgement Receipt will establish the international filing date of the application.

Doc Code: DIST.E.FILE **Document Description: Electronic Terminal Disclaimer - Filed**	PTO/SB/25 U.S. Patent and Trademark Office Department of Commerce

Electronic Petition Request	**TERMINAL DISCLAIMER TO OBVIATE A PROVISIONAL DOUBLE PATENTING REJECTION OVER A PENDING "REFERENCE" APPLICATION**
Application Number	14067620
Filing Date	30-Oct-2013
First Named Inventor	Brian Maples
Attorney Docket Number	30171-0025002 / ITI-001
Title of Invention	Nicking and Extension Amplification Reaction for the Exponential Amplification of Nucleic Acids

☒ Filing of terminal disclaimer does not obviate requirement for response under 37 CFR 1.111 to outstanding Office Action

☒ This electronic Terminal Disclaimer is not being used for a Joint Research Agreement.

Owner	Percent Interest
Ionian Technologies, Inc.	100%

The owner(s) of percent interest listed above in the instant application hereby disclaims, except as provided below, the terminal part of the statutory term of any patent granted on the instant application which would extend beyond the expiration date of the full statutory term of any patent granted on pending reference Application Number(s)

12173020 filed on 07/14/2008

14067623 filed on 10/30/2013

11778018 filed on 07/14/2007

as the term of any patent granted on said reference application may be shortened by any terminal disclaimer filed prior to the grant of any patent on the pending reference application. The owner hereby agrees that any patent so granted on the instant application shall be enforceable only for and during such period that it and any patent granted on the reference application are commonly owned. This agreement runs with any patent granted on the instant application and is binding upon the grantee, its successors or assigns.

In making the above disclaimer, the owner does not disclaim the terminal part of any patent granted on the instant application that would extend to the expiration date of the full statutory term of any patent granted on said reference application, "as the term of any patent granted on said reference application may be shortened by any terminal disclaimer filed prior to the grant of any patent on the pending reference application," in the event that any such patent granted on the pending reference application: expires for failure to pay a maintenance fee, is held unenforceable, is found invalid by a court of competent jurisdiction, is statutorily disclaimed in whole or terminally disclaimed under 37 CFR 1.321, has all claims canceled by a reexamination certificate, is reissued, or is in any manner terminated prior to the expiration of its full statutory term as shortened by any terminal disclaimer filed prior to its grant.

◉ Terminal disclaimer fee under 37 CFR 1.20(d) is included with Electronic Terminal Disclaimer request.

○ I certify, in accordance with 37 CFR 1.4(d)(4), that the terminal disclaimer fee under 37 CFR 1.20(d) required for this terminal disclaimer has already been paid in the above-identified application.

Applicant claims the following fee status:

○ Small Entity

○ Micro Entity

◉ Regular Undiscounted

I hereby declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code and that such willful false statements may jeopardize the validity of the application or any patent issued thereon.

THIS PORTION MUST BE COMPLETED BY THE SIGNATORY OR SIGNATORIES

I certify, in accordance with 37 CFR 1.4(d)(4) that I am:

◉ An attorney or agent registered to practice before the Patent and Trademark Office who is of record in this application

Registration Number 59749

○ A sole inventor

○ A joint inventor; I certify that I am authorized to sign this submission on behalf of all of the inventors as evidenced by the power of attorney in the application

○ A joint inventor; all of whom are signing this request

Signature	/Ian J.S. Lodovice, Reg. No. 59,749/
Name	Ian J. Lodovice

*Statement under 37 CFR 3.73(b) is required if terminal disclaimer is signed by the assignee (owner).
Form PTO/SB/96 may be used for making this certification. See MPEP § 324.

Electronic Patent Application Fee Transmittal

Application Number:	14067620
Filing Date:	30-Oct-2013
Title of Invention:	Nicking and Extension Amplification Reaction for the Exponential Amplification of Nucleic Acids
First Named Inventor/Applicant Name:	Brian K. Maples
Filer:	Ian J.S. Lodovice/Mary Florczak
Attorney Docket Number:	30171-0025002 / ITI-001

Filed as Large Entity

Filing Fees for Utility under 35 USC 111(a)

Description	Fee Code	Quantity	Amount	Sub-Total in USD($)
Basic Filing:				
Statutory or Terminal Disclaimer	1814	1	160	160
Pages:				
Claims:				
Miscellaneous-Filing:				
Petition:				
Patent-Appeals-and-Interference:				
Post-Allowance-and-Post-Issuance:				

Description	Fee Code	Quantity	Amount	Sub-Total in USD($)
Extension-of-Time:				
Miscellaneous:				
		Total in USD ($)		**160**

Doc Code: DISQ.E.FILE
Document Description: Electronic Terminal Disclaimer – Approved

Application No.: 14067620

Filing Date: 30-Oct-2013

Applicant/Patent under Reexamination: Maples et al.

Electronic Terminal Disclaimer filed on September 2, 2016

☒ APPROVED

This patent is subject to a terminal disclaimer

☐ DISAPPROVED

Approved/Disapproved by: Electronic Terminal Disclaimer automatically approved by EFS-Web

U.S. Patent and Trademark Office

Electronic Acknowledgement Receipt	
EFS ID:	26797180
Application Number:	14067620
International Application Number:	
Confirmation Number:	4288
Title of Invention:	Nicking and Extension Amplification Reaction for the Exponential Amplification of Nucleic Acids
First Named Inventor/Applicant Name:	Brian K. Maples
Customer Number:	26161
Filer:	Ian J.S. Lodovice/Mary Florczak
Filer Authorized By:	Ian J.S. Lodovice
Attorney Docket Number:	30171-0025002 / ITI-001
Receipt Date:	02-SEP-2016
Filing Date:	30-OCT-2013
Time Stamp:	12:55:15
Application Type:	Utility under 35 USC 111(a)

Payment information:

Submitted with Payment	yes
Payment Type	Deposit Account
Payment was successfully received in RAM	$160
RAM confirmation Number	48
Deposit Account	061050
Authorized User	Florczak, Mary
The Director of the USPTO is hereby authorized to charge indicated fees and credit any overpayment as follows:	

File Listing:

Document Number	Document Description	File Name	File Size(Bytes)/ Message Digest	Multi Part /.zip	Pages (if appl.)
1	Electronic Terminal Disclaimer-Filed	eTerminal-Disclaimer.pdf	35211 b6c230d5f7ea66504ab8c672e35df9f9bf89568d	no	2
Warnings:					
Information:					
2	Fee Worksheet (SB06)	fee-info.pdf	30532 7a6a9cc5b70d9d8ee3b752c4744f48f335ee364a	no	2
Warnings:					
Information:					
		Total Files Size (in bytes):	65743		

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New Applications Under 35 U.S.C. 111
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National Stage of an International Application under 35 U.S.C. 371
If a timely submission to enter the national stage of an international application is compliant with the conditions of 35 U.S.C. 371 and other applicable requirements a Form PCT/DO/EO/903 indicating acceptance of the application as a national stage submission under 35 U.S.C. 371 will be issued in addition to the Filing Receipt, in due course.

New International Application Filed with the USPTO as a Receiving Office
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IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

First Named Inventor	: Brian K. Maples	Art Unit	:	1637
Serial No.	: 14/067,620	Examiner	:	Angela M. Bertagna
Filed	: October 30, 2013	Conf. No.	:	4288

Title : NICKING AND EXTENSION AMPLIFICATION REACTION FOR THE EXPONENTIAL AMPLIFICATION OF NUCLEIC ACIDS

Commissioner for Patents
P.O. Box 1450
Alexandria, VA 22313-1450

AMENDMENT AND REPLY TO ACTION OF FEBRUARY 11, 2016

Please consider the following amendment and reply.

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

First Named Inventor	:	Brian K. Maples	Art Unit	:	1637
Serial No.	:	14/067,620	Examiner	:	Angela M. Bertagna
Filed	:	October 30, 2013	Conf. No.	:	4288

Title : NICKING AND EXTENSION AMPLIFICATION REACTION FOR THE EXPONENTIAL AMPLIFICATION OF NUCLEIC ACIDS

Commissioner for Patents
P.O. Box 1450
Alexandria, VA 22313-1450

AMENDMENT AND REPLY TO ACTION OF FEBRUARY 11, 2016

Please consider the following amendment and reply.

List of claims (replacing prior versions).

1. - 66. (Canceled)

67. (Currently Amended) A method of amplifying a target polynucleotide sequence ~~of a target nucleic acid present in a sample obtained from an animal~~, the method comprising:
(a) obtaining, from an animal, a sample comprising a target nucleic acid, the target nucleic acid comprising the target polynucleotide sequence,

~~(a)~~ (b) ~~preparing,~~ without first subjecting the target nucleic acid to a thermal denaturation step associated with amplification of the target polynucleotide sequence, combining, in a single step, the obtained sample directly with an amplification reagent mixture or diluting the obtained sample and combining, in a single step, the diluted sample with ~~a~~ an amplification reagent mixture, in either case, the amplification reagent mixture being free of bumper primers and comprising:

(i) ~~the target nucleic acid comprising the target polynucleotide sequence,~~
~~(ii)~~ a polymerase,
~~(iii)~~ (ii) a nicking enzyme,
~~(iv)~~ (iii) a first oligonucleotide comprising a nicking site and a nicking enzyme binding site, and
~~(v)~~ (iv) a second oligonucleotide comprising a nicking site and a nicking enzyme binding site, ~~and~~

~~(b)~~ (c) subjecting the reaction mixture formed by the step of combining to essentially isothermal conditions to amplify the target polynucleotide sequence, and

(d) detecting the amplified target polynucleotide sequence in real time within 10 minutes of subjecting the reaction mixture to essentially isothermal conditions.

68. (Previously Presented) The method of claim 67, wherein the target polynucleotide sequence is amplified from steps comprising:

(a) forming a first duplex comprising the target polynucleotide sequence and the first oligonucleotide;

(b) extending, using the polymerase, the first oligonucleotide along the target polynucleotide sequence to form an extended first oligonucleotide comprising a sequence complementary to the second oligonucleotide;

(c) forming a second duplex comprising the second oligonucleotide and the extended first oligonucleotide;

(d) extending, using the polymerase, the second oligonucleotide along the extended first oligonucleotide to form a third duplex comprising an extended second oligonucleotide comprising a sequence complementary to the first oligonucleotide and a first double-stranded nicking enzyme binding site;

(e) nicking, with the nicking enzyme, the first nicking site on the third duplex to produce a fourth duplex comprising the extended second oligonucleotide and a fragment of the extended first oligonucleotide; and

(f) extending, using the polymerase, the fragment of the extended first oligonucleotide along the extended second oligonucleotide of the fourth duplex to produce a double-stranded nucleic acid product and a second double-stranded nicking enzyme binding site.

69. (Previously Presented) The method of claim 68, wherein the double-stranded nucleic acid product comprises:

i) a first strand and a second strand, wherein the first strand comprises a first polynucleotide sequence corresponding to the target polynucleotide sequence and the second strand comprises a second polynucleotide sequence complementary to the target polynucleotide sequence, and

ii) first and second double-stranded nicking sites spaced apart by the target polynucleotide sequence.

70. (Previously Presented) The method of claim 68, further comprising the steps of:

a) nicking, using the nicking enzyme, the first nicking site of the double-stranded nucleic acid product to produce a fifth duplex comprising a first polynucleotide sequence corresponding to the target polynucleotide sequence and a fragment of the first oligonucleotide, and nicking, using the nicking enzyme, the second nicking site of the double-stranded nucleic acid product to

produce a sixth duplex comprising a second polynucleotide sequence complementary to the target polynucleotide sequence and a fragment of the second oligonucleotide;

b) extending, using the polymerase, the fragment of the first oligonucleotide along the first polynucleotide sequence of the fifth duplex to produce a first double stranded product comprising a copy of the nicking site and a copy of the first polynucleotide sequence and extending, using the polymerase, the fragment of the second oligonucleotide along the second polynucleotide sequence of the sixth duplex to produce a second double stranded product comprising a copy of the nicking site and a copy of the second polynucleotide sequence; and

c) nicking, using the nicking enzyme, the copy of the nicking site of the first double stranded product to release a copy of the first polynucleotide sequence and nicking, using the nicking enzyme, the copy of the nicking site of the second double stranded product to release a copy of the second polynucleotide sequence.

71. (Previously Presented) The method of claim 67, wherein the animal is a human.

72. (Previously Presented) The method of claim 67, wherein the target nucleic acid is obtained from an animal pathogen.

73. (Previously Presented) The method of claim 72, wherein the animal pathogen is a single-stranded DNA virus, double-stranded DNA virus, or single-stranded RNA virus.

74. (Withdrawn) The method of claim 72, wherein the animal pathogen is a bacterium.

75. (Withdrawn) The method of claim 72, wherein the animal pathogen contains spores and the target polynucleotide is amplified from the spores without the need for lysis of the spores.

76. (Currently Amended) The method of claim 67, wherein the sample obtained from an animal is ~~obtained from the~~ blood, ~~bone marrow,~~ mucus, ~~lymph, hard tissues, biopsies,~~ sputum, saliva, tears, feces ~~faeces~~ or urine of the animal.

77. (Currently Amended) The method of claim 76, wherein the sample obtained from an animal is ~~obtained from the~~ mucus, sputum, or saliva of the animal.

78. (Withdrawn) The method of claim 67, wherein the target nucleic acid is double-stranded DNA.

79. (Withdrawn) The method of claim 67, wherein the target nucleic acid is single-stranded DNA.

80. (Previously Presented) The method of claim 67, wherein the target nucleic acid is RNA.

81. (Withdrawn) The method of claim 67, wherein the target nucleic acid is selected from the group consisting of genomic DNA, plasmid DNA, viral DNA, mitochondrial DNA, cDNA, synthetic double-stranded DNA and synthetic single-stranded DNA.

82. (Withdrawn) The method of claim 81, wherein the target nucleic acid is genomic DNA.

83. (Previously Presented) The method of claim 67, wherein the target nucleic acid is viral DNA or viral RNA.

84. (Canceled)

85. (Previously Presented) The method of claim 67, wherein the nicking enzyme is Nt.BstNBI.

86. (Previously Presented) The method of claim 67, wherein the nicking enzyme does not nick within the target polynucleotide sequence.

87. (Previously Presented) The method of claim 67, wherein amplification of the target polynucleotide sequence is performed without the use of temperature cycling.

88. (Previously Presented) The method of claim 67, wherein amplification of the target polynucleotide sequence is performed at about 55°C-59°C.

89. (Canceled)

90. (Previously Presented) The method of claim 68, which is performed at a temperature higher than the melting temperature of the first oligonucleotide/target polynucleotide sequence complex.

91. (Canceled)

92. (Currently Amended) The method of claim 67 ~~91~~, wherein the amplification product is detected by a detection method selected from the group consisting of ~~gel electrophoresis, mass spectrometry~~, fluorescence, intercalating dye detection, fluorescence resonance energy transfer (FRET), molecular beacon detection, ~~surface capture,~~ ~~capillary electrophoresis,~~ and incorporation of labeled nucleotides~~ to allow detection by capture, fluorescence polarization, and lateral flow capture, or a combination thereof~~.

93. (Canceled)

94. (Canceled)

95. (Withdrawn, Currently Amended) A method of amplifying a target polynucleotide sequence~~ of genomic DNA present in a sample obtained from an animal~~, the method comprising:

(a) obtaining, from an animal, a sample containing genomic DNA, the genomic DNA comprising a target nucleic acid, the target nucleic acid comprising the target polynucleotide sequence,

(b) ~~preparing,~~ without first subjecting the genomic DNA to a thermal denaturation step associated with amplification of the target polynucleotide sequence, combining, in a single step, the obtained sample directly with a reaction mixture or diluting the obtained sample and combining, in a single step, the diluted sample with ~~a~~ an amplification reagent mixture, in either case, the amplification reagent mixture being free of bumper primers and comprising:

(i) ~~the genomic DNA comprising the target polynucleotide sequence,~~

~~(ii)~~ a polymerase,

~~(iii)~~ (ii) a nicking enzyme,

~~(iv)~~ (iii) a first oligonucleotide comprising a nicking site and a nicking enzyme binding site, ~~and~~

~~(v)~~ (iv) a second oligonucleotide comprising a nicking site and a nicking enzyme binding site, and

~~b)~~ (c) subjecting the reaction mixture formed by the step of combining to essentially isothermal conditions to amplify the target polynucleotide sequence, and

(d) detecting the amplified target polynucleotide sequence in real time within 10 minutes of subjecting the reaction mixture to essentially isothermal conditions.

96. (Previously Presented) The method of claim 67, wherein the target polynucleotide sequence is amplified about 1E+8-fold.

97. (Previously Presented) The method of claim 67, wherein the target polynucleotide sequence is amplified about 3E+9-fold.

98. (Previously Presented) The method of claim 67, wherein the target polynucleotide sequence is amplified about 7E+10-fold.

99. (Previously Presented) The method of claim 67, wherein amplification of the target polynucleotide sequence is performed at a constant temperature for about 1 to 12 minutes

100. (Previously Presented) The method of claim 67, wherein amplification of the target polynucleotide sequence is performed at a constant temperature for about 1 to 10 minutes.

101. (Previously Presented) The method of claim 67, wherein amplification of the target polynucleotide sequence is performed at a constant temperature for about 1 to 8 minutes.

102. (Previously Presented) The method of claim 67, wherein amplification of the target polynucleotide sequence is performed at a constant temperature for about 1 to 5 minutes.

103. (Previously Presented) The method of claim 67, wherein amplification of the target polynucleotide sequence is performed at a constant temperature for about 1 to 2.5 minutes.

104. (Previously Presented) The method of claim 67, wherein amplification of the target polynucleotide sequence is performed at a constant temperature for about 2.5 to 5 minutes.

105. (Previously Presented) The method of claim 67, wherein amplification of the target polynucleotide sequence is performed at a constant temperature for about 2.5 to 8 minutes.

106. (Previously Presented) The method of claim 67, wherein amplification of the target polynucleotide sequence is performed at a constant temperature for about 2.5 to 10 minutes.

107. (Currently Amended) A method of amplifying a target polynucleotide sequence ~~of a target nucleic acid present in a sample obtained from an animal~~, the method comprising:

(a) obtaining, from an animal, a sample comprising a target nucleic acid, the target nucleic acid comprising the target polynucleotide sequence,

~~(a)~~ (b) ~~preparing,~~ without first subjecting the target nucleic acid to a thermal denaturation step associated with amplification of the target polynucleotide sequence, combining, in a single step, the obtained sample directly with a reaction mixture or diluting the obtained sample and combining, in a single step, the diluted sample with ~~a~~ an amplification reagent mixture, in either case, the amplification reagent mixture being free of bumper primers and comprising:

(i) ~~the target nucleic acid comprising the target polynucleotide sequence,~~

~~(ii)~~ a polymerase,

~~(iii)~~ (ii) a nicking enzyme,

~~(iv)~~ (iii) a first oligonucleotide comprising a nicking site and a nicking enzyme binding site, and

~~(v)~~ (iv) a second oligonucleotide comprising a nicking site and a nicking enzyme binding site, ~~and~~

~~(b)~~ (c) subjecting the reaction mixture formed by the step of combining to essentially isothermal conditions to amplify the target polynucleotide sequence,

(d) detecting the amplified target polynucleotide sequence in real time within 10 minutes of subjecting the reaction mixture to essentially isothermal conditions, and

wherein the target polynucleotide sequence is amplified about 1E+8-fold in about 1 to 12 minutes.

REMARKS

Upon entry of the above amendment, claims 67-83, 85-88, 90, 92 and 95-107 will be pending. Claims 74-75, 78-79, 81-82 and 95 were previously withdrawn, so are not currently under examination. Claims 1-67, 84, 89 and 93-94 were previously canceled. Claim 91 is newly canceled, and claims 67, 76, 77, 92, 95 and 107 have been amended. Most of the amendments are simply to clarify scope and are supported throughout the specification so that no new matter is introduced. For example, the amendments to claims 67, 95 and 107 find support at page 18, lines 3-5 and 12-13; page 26, lines 15-17; page 36, lines 29-30; page 32, line 25; Figures 6-8, and the Examples.

Reconsideration and allowance of the claims are respectfully requested in view of the above amendments and the following remarks.

Claim Rejection Under 35 USC § 112, First Paragraph (New Matter)

Claim 107 stands rejected under the first paragraph of 35 U.S.C. § 112 for allegedly failing to comply with the written description requirement. This contention is traversed for the reasons outlined in Applicants response filed on May 27, 2015. However, without conceding the appropriateness of the present rejection, and solely in the interest of advancing prosecution, claim 107 has been amended to recite "without first subjecting the target nucleic acid to a thermal denaturation step associated with amplification of the target polynucleotide sequence." Reconsideration and withdrawal of the rejection of under 35 U.S.C. 112, first paragraph is requested.

First Claim Rejection Under 35 USC § 103

Claims 67-73, 76, 77, 80, 83, 85-88, 90-92, and 96-98 stand rejected under pre-AIA 35 U.S.C. 103(a) as allegedly being unpatentable over Wick et al. (US 6,063,604)("Wick") in view of Kong et al. (US 6,191,267)("Kong"). Applicant traverses this rejection at least for the reason that the combination of cited references fails to teach or suggest each and every feature of the claims as amended.

As amended, claim 67 requires the step of combining, in a single step, a sample or diluted sample with an amplification reagent mixture that includes: (i) a polymerase, (ii) a nicking enzyme, (iii) a first oligonucleotide comprising a nicking site and a nicking enzyme binding site, and (iv) a second oligonucleotide comprising a nicking site and a nicking enzyme binding site. The reaction mixture that is formed by the step of "combining, in a single step" is a mixture that includes a sample or diluted sample, a polymerase, a nicking enzyme, and two oligonucleotides, each comprising a nicking site and a nicking enzyme binding site.

The claims as amended also require detecting amplified target polynucleotide sequences in real time within 10 minutes of subjecting the reaction mixture to essentially isothermal conditions. Detection of amplification products within 10 minutes of subjecting the reaction mixture to essentially isothermal reaction conditions avoids prolonged incubation periods between the sample (or diluted sample) and amplification reagents. Detection in real time avoids a separate detection step, as occurs in Wick (discussed below), following completion of the amplification reaction.

Further, the claims as amended require the amplification reagent mixture to be free of bumper primers. In accordance with the invention, amplification of the target nucleic acid proceeds in the absence of bumper primers.

In contrast to the claims as amended, Wick requires at least two separate steps to form a reaction mixture including a target, primers and enzymes. In the method taught by Wick, one step is to combine the target nucleic acid with primers. Wick, col. 28, lines 66-67. The resulting mixture is subjected to thermal denaturation. Id. After a period of time to cool the target/primer reaction mixture, Wick teaches an additional step of adding the remaining amplification reagents, including the enzymes, to the cooled sample. Wick, col. 29, lines 1-7. See also Wick, col. 30, lines 22-31; col. 31, lines 1-12; col. 32, lines 15-24.

As was known to one of ordinary skill at the time of the invention, heating and then cooling a target nucleic acid in the presence of primers allows for denaturation of the target followed by substantial annealing of the target with the primers. Moreover, adding enzymes in a separate step after the heating/cooling step preserves the viability of the enzymes, which would otherwise be destroyed.

Even if one were to substitute pH denaturation for the thermal denaturation of Wick, one of skill in the art would still have performed such pH denaturation in the presence of primers to achieve substantial annealing of the primers with the target. Moreover, enzymes would still need to be added in a subsequent step to avoid subjecting the enzymes to the extreme pH required for denaturation. Accordingly, even if the thermal denaturation of Wick was replaced by pH denaturation, such a method would still require at least two separate steps: pH denaturation of a mixture including the target and primers and, subsequently, the combining of the pH-denatured mixture and enzymes.

In further contrast to the claims as amended, Wick fails to disclose or suggest the step of detecting amplified target polynucleotide sequences in real time within 10 minutes of subjecting the reaction mixture to essentially isothermal conditions. First, Wick requires amplification incubation times of at least 60 minutes under isothermal conditions. Wick, col. 29, line 8; col. 30, lines 31-32; col. 31, lines 13-16; col. 32, lines 24-25. Moreover, Wick teaches detection methods such as visualization or autoradiograph detection of electrophoretically separated amplicon mixtures on polyacrylamide gels. Wick, col. 21, lines 28-35; col. 29, lines 11-44. Such methods are not real-time methods and do not occur with 10 minutes of subjecting the mixture to essentially isothermal conditions.

Kong fails to remedy the deficiencies of Wick. Kong also requires at least two separate steps to form a reaction mixture including a target, primers and enzymes. One step taught by Kong is combining target DNA and primers in mix A, which is subsequently heated. Following a cooling incubation period "to allow annealing of the primers [with the target]," a separate mixture containing the enzymes, mix B, is added to mix A. Kong, col. 14, lines 20-56.

Additionally, Kong also fails to disclose or suggest the step of detecting amplified target polynucleotide sequences in real time within 10 minutes of subjecting the reaction mixture to essentially isothermal conditions. Kong requires an amplification incubation time of at least 20 minutes (Kong, col. 14, lines 57-58) and detection of amplification products by separate, post-amplification gel electrophoresis (Kong, col. 14, lines 61-64).

For at least these reasons, independent claim 67 is patentable in view of the combination of Wick and Kong. Because independent claim 67 is patentable over Wick and Kong, dependent

claims 68-73, 76, 77, 80, 83, 85-88, 90-92, and 96-98 are also patentable over Wick and Kong. For at least these reasons, Applicant requests reconsideration and withdrawal of this rejection.

Second Claim Rejection Under 35 USC § 103

Claims 99-107 stand rejected under pre-AIA 35 U.S.C. 103(a) as allegedly being unpatentable over Wick et al. (US 6,063,604)("Wick") in view of Kong et al. (US 6,191,267)("Kong") and further in view of Yao et al. (US 2008/0096257)("Yao").

As discussed above, amended claim 67 is patentable in view of the combination of Wick and Kong. Because independent claim 67 is patentable over Wick and Kong, dependent claims 99-106 are also patentable over Wick and Kong. As with independent claim 67, independent claim 107 requires the step of combining, in a single step, a sample or diluted sample with an amplification reagent mixture that includes: (i) a polymerase, (ii) a nicking enzyme, (iii) a first oligonucleotide comprising a nicking site and a nicking enzyme binding site, and (iv) a second oligonucleotide comprising a nicking site and a nicking enzyme binding site; and detecting amplified target polynucleotide sequences in real time within 10 minutes of subjecting the reaction mixture to essentially isothermal conditions. Thus, for at least the reasons discussed above, independent claim 107 is patentable over Wick and Kong. Yao fails to cure the deficiencies of Wick and Kong, at least for the reason that the combination of cited references fails to teach or suggest each and every feature of the claims as amended.

The SDA methods of Yao require purification of human genomic DNA from a sample prior to amplification of a target sequence in the DNA. Yao, paras. [0034], [0037], [0038], [0042] and [0046]. It would have been known to the skilled artisan that Yao's purification of the target would concentrate the target nucleic acid and substantially separate the target nucleic acid from inhibitors and background materials present in a sample obtained from an animal, thereby facilitating amplification of the target nucleotide sequence. In contrast, the present claims require the step of combining, in a single step, a sample or diluted sample with the as defined amplification reagent mixture. The skilled artisan would appreciate that dilution may occur in the context of, for example, a buffer suitable to lyse cells in a sample, without concentrating the target nucleic acid or substantially removing any inhibitors and background materials present in a sample obtained from an animal, as occurs in the purification of Yao. The SDA methods

taught by Yao also require bumper primers. Yao, paras. [0036]-[0038], Table 1. At the time of the invention, the skilled artisan would have known that SDA was routinely performed with bumper primers to facilitate amplification. As described in a textbook excerpt from 2003 (Exhibit A), the addition of bumper primers was a "preferred solution" for displacing original primers annealed to the target. (Artificial DNA: Methods and Applications (2003), Khudyakov Y.E. & Fields, H.A. (Eds), Synthetic DNA Used in Amplification Reactions (pp. 127-130), CRC Press LLC., attached as Exhibit A).

Accordingly, one of skill in the art would not have modified any combination of Wick and Kong (whether or not in view of Yao) to detect in real time within 10 minutes of subjecting the reaction mixture to essentially isothermal conditions without incorporating the target purification and bumper primers required by Yao. As understood in the art, purification and bumper primers facilitated SDA reactions and there would have been no expectation of success for the claimed invention in their absence.

Accordingly, even if Wick, Kong and Yao are combined in a manner suggested by the Office, such a method would require purification of the target and bumper primers. In contrast, the present claims require obtaining a sample from an animal and combining the obtained sample either directly, or after dilution, with amplification reagents. In further contrast, the present claims also require a reaction mixture that is free of bumper primers.

For at least these reasons, claims 99-107 are patentable over Wick in view of Kong and further in view of Yao. Applicant requests reconsideration and withdrawal of this rejection.

Double Patenting

Claims 67-73, 76, 77, 80, 83, 85-88, 90-92, and 96-106 stand provisionally rejected on the ground of nonstatutory double patenting as being allegedly unpatentable over claims 67-69, 74-76, 79, 80, 83, 85, 87-90, 92-94, and 97-106 of copending Application No. 14/067,623.

Claim 107 stands provisional rejected on the ground of nonstatutory double patenting as being allegedly unpatentable over claims 67-69, 74-76, 79, 80, 83, 85, 87-90, 92-94, and 97-106 of copending Application No. 14/067,623 in view of Yao.

Claims 67-73, 76, 77, 80, 83, 85-88, 90-92, and 96-106 stand provisionally rejected on the ground of nonstatutory double patenting as being allegedly unpatentable over claims 1-9, 12, 14-17, 19-41, and 44- 46 of copending Application No. 12/173,020.

Claim 107 stands provisionally rejected on the ground of nonstatutory double patenting as being allegedly unpatentable over claims 1-9, 12, 14-17, 19-41, and 44- 46 of copending Application No. 12/173,020 in view of Yao.

Claims 67-73, 76, 77, 80, 83, 86-88, 90-92, and 96-98 stand provisionally rejected on the ground of nonstatutory double patenting as being allegedly unpatentable over claims 125-130 of copending Application No. 11/778,018 in view of Wick.

Claims 99-107 stand provisionally rejected on the ground of nonstatutory double patenting as being allegedly unpatentable over claims 125-130 of copending Application No. 11/778,018 in view of Wick and further in view of Yao.

Claim 85 stands provisionally rejected on the ground of nonstatutory double patenting as being allegedly unpatentable over claims 125-130 of copending Application No. 11/778,018 in view of Wick and further in view of Kong.

Applicant does not concede that the rejections above are appropriate. Further, as the applications cited above are all currently pending, Applicant requests that the rejections be held in abeyance pending the identification of allowable subject matter.

CONCLUSION

In light of the arguments made herein, applicants submit that the pending claims are patentable and request early and favorable action thereon. If any issues remain, the Examiner is asked to telephone the Applicants' representative Ian Lodovice at 617-956-5972 to arrange a time for an interview.

Applicants do not concede any positions of the Office that are not expressly addressed above, nor do applicants concede that there are not other good reasons for patentability of the presented claims or other claims.

This response is being filed with a Three-Month extension of time in the amount of $1,400.00 on the Electronic Filing System. Apply those fees and any other necessary charges or credits to Deposit Account 06-1050, referencing the above attorney docket number.

Respectfully submitted,

Date: August 11, 2016______________

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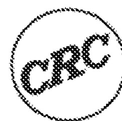
EXHIBIT A

Artificial DNA

Methods and Applications

Edited by
Yury E. Khudyakov
Howard A. Fields



CRC PRESS
Boca Raton London New York Washington, D.C.

Library of Congress Cataloging-in-Publication Data

Artificial DNA : methods and applications / edited by Yury E. Khudyakov and Howard A. Fields.
p. cm.
Includes bibliographical references and index.
ISBN 0-8493-1426-7 (alk. paper)
1. DNA—Synthesis. 2. DNA—Derivatives. 3. DNA probes. 4. Genetic engineering. I. Khudyakov, Yury E. II. Fields, Howard A.

QP624 .A785 2002
660.6'5—dc21 2002067416

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No claim to original U.S. Government works
International Standard Book Number 0-8493-1426-7
Library of Congress Card Number 2002067416
Printed in the United States of America 1 2 3 4 5 6 7 8 9 0
Printed on acid-free paper

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The linker sequence allows for the binding of primers and detection tags. Primers work best with a melting temperature slightly greater than the reaction temperature. The primers may be allele-specific if desired. If using two primers, one primer is complementary to the circle; the other has the same sequence of a different part of the linker region of the circle. Obviously, the primers should not complement each other.

RCA-CACHET is interesting in that the second ligation probe has two 3′ ends, one for ligation and one for polymerization.[101] The purpose is to allow the bound primer to expose a 5′ end, thus decreasing nonspecific polymerization without ligation. The circles used are preformed with a complementary helper oligonucleotide, which forces the ends together for ligation.

4.2.3.3 Conclusions

Undoubtedly, a polymerase running circuits around synthetic circles can generate tremendous amounts of DNA in a short time, especially with ramification. Although exponential amplification allows single-copy genes to be detected with a sensitivity of about 17,000 molecules,[103] the greatest amplification comes from artificial templates,[104] with sensitivities down to ten copies. However, this sensitivity is not realized if the circle lacks free rotation by being padlocked onto a template. It can move linearly down the template[86] a few hundred nucleotides, but polymerization with the usually processive Phi29 polymerase is sterically inhibited if an end is too distant.[106] However, recently conditions for exponential amplification have been described with an alternate polymerase (from *Bacillus stearothemophilus)* that escapes these topological constraints observed with the original polymerase,[107] allowing sensitive detection down to ten target molecules.[108] Blocked DNA amplification *in situ* has been overcome by digesting the nontarget DNA with restriction enzymes and endonucleases.[109]

Currently, linear rolling-circle amplification appears its most popular use. Linear amplification may be sufficiently sensitive if a detection molecule is incorporated for each repeat. The major benefits of RCA are the allele specificity granted by the ligation and the multiplexing allowed by surface amplification such as on a microarray.[110,111] RCA on microarrays may be used for direct amplification of target sequence[112] or as a signal amplification method from targets previously amplified by PCR.[113,114] Conjugating a primer to an antibody allows signal amplification for sensitive protein detection[115,116] or *in situ* localization of mRNA hybridized to hapten-labeled DNA probes.[117]

4.2.4 Strand-Displacement Amplification

RCA uses strand displacement for exponential amplification. However, each new upstream strand made is eventually suffocated by the closure of a double strand. Given that the original strand can be kilobases long, the amplification is substantial. For amplifying only short linear sequences, a method is needed for reviving the template for reuse. PCR reclaims its template through heat denaturation. Strand-displacement amplification (SDA) uses a restriction enzyme to restore the double-stranded templates during the isothermal reaction.

4.2.4.1 Reaction Mechanics

SDA is continuous and exponential amplification of target sequence between two primers using a restriction enzyme, an exonuclease-deficient polymerase, one thiolated nucleotide, and three unmodified nucleotides[118] (Figure 4.5A). The restriction enzyme nicks the unprotected strand of a hemithiolated asymmetrical recognition site introduced from a primer upstream of the target sequence. The polymerase commences nucleotide addition at the nick, copying a new target strand while displacing its predecessor. The 3′ end of the displaced strand binds a complementary primer and is then extended to regenerate a double-stranded but half-shielded restriction site. This begins a new cycle of primer extension, cleavage, and displacement. The cycling occurs continuously, not

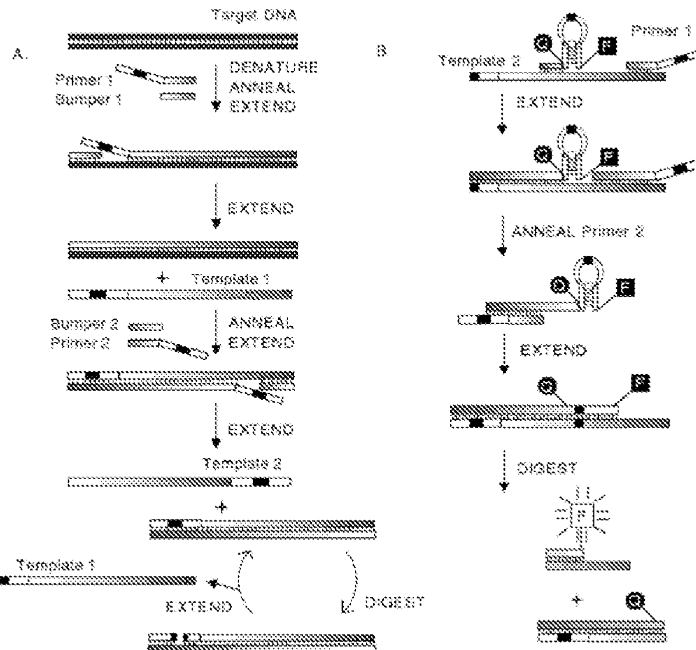

FIGURE 4.5 Strand displacement amplification. (A) Amplification from only one strand of target DNA is shown. A primer containing a Type IIs restriction enzyme (REN) site anneals and is extended with thiolated nucleotides by polymerase. A bumper primer dislodges the single-stranded Template 1. Template 1 binds Primer 2 and Bumper 2, resulting in free template 2 and a double-stranded REN site protected on the new strand from cleavage by the thiolated nucleotides. The REN digests the primer, generating an opening for polymerase extension and strand displacement. Released Template 1 can reenter the cycle by binding Primer 2, and free Template 2 undergoes similar amplification with Primer 1 (not shown). (B) Real-time detection. A hairpin probe with a quenched fluorophore and a REN site in its loop binds downstream of Primer 1. The probe is elongated and then displaced by primer extension. The elongation of the probe complements and binds Primer 2, whose extension straightens the hairpin. Due to its designed sequence, neither strand of the double-stranded REN site of the loop is protected from digestion, and fluorescence is released.

discretely, and each displaced strand from an extended primer functions as a template for the alternate primer. Thus, the reaction is self-perpetuating, exhausting only when the enzymes are no longer in excess.[119]

Only the 3′ end of the primer is specific to the target, yet amplification requires that the 5′ end of the primer, containing the restriction enzyme site, become double-stranded. Displaced strands generated during amplification have their 3′ ends extended upon primer binding to form the restriction site. However, the 5′ end of the primers that bind to the original template have no inherent means of becoming double-stranded. A mechanism had to be designed to displace the original binders and introduce a double-stranded restriction enzyme site to initiate the reaction. Originally, the target was first trimmed with another restriction enzyme to generate known extendible ends to serve as the target-specific 3′ annealing site.[118] This procedure was time-consuming and restrictive because the target had to be selected between appropriate restriction sites and only generated million-fold amplification in 4 h. A preferred solution was devised by adding bumper primers.[120] These primers bind specifically just upstream of the amplification primers. They do not necessarily

contain a restriction site and are at about one tenth the concentration of the amplification primers, which must extend first. When the bumper primers are extended, they displace the amplification primers, launching the self-sustaining cycle. This allows 100-million-fold amplification in just 2 h.

The amplification was immense; however, the background was so high that it prevented the products from being directly detected on a gel. The high background was aggravated by the low stringency possible with mesophilic enzymes. Short primer-dimers made 3′ of the restriction site are amplified very efficiently, much more than target sequences. For example, mesophilic strand displacement with the polymerase Klenow exo- loses tenfold efficiency with every 50 nucleotides of target span.[120] Background is decreased by using thermophilic enzymes at about 50 to 65°C,[121,122] which allows more specific primer binding. In addition, the efficiency of thermophilic SDA increases to produce more than 1-billion-fold amplification in 15 min. The reaction can be used for detection of RNA only by first preparing cDNA.[123,124]

4.2.4.2 Oligonucleotides

4.2.4.2.1 Primers

There are four primers in the current SDA system. The internal primers are at tenfold higher concentration than the external primers. The external primers are only incorporated during the first round in order to bump the internal primers and give defined ends to the target.[125] The 3′ ends of the primers must not form primer-dimers because any made 3′ to the restriction site will be amplified exponentially, probably even more efficiently than the product. The 5′ ends of the primers are not sequence specific, but must be long enough to stabilize the restriction enzyme on the succeeding nonpalindromic restriction enzyme site.[124] A nonpalindromic enzyme must be used so that the thiolated nucleotide will protect only one strand. A symmetrical site would grant protection to all strands made after original target cleavage, halting the reaction. Obviously, the target sequence between the primers cannot contain the restriction enzyme recognition site.

4.2.4.2.2 Detection

In the beginning, the promiscuity of the mesophilic reaction necessitated probing with a labeled specific probe. Desired products were separated from the rubbish by a specific solid-phase capture oligonucleotide. Alkaline phosphatase-labeled detection probes then bound to impart a sensitive signal.[126] Capturing the amplicons on beads and incorporating DIG-dUTP in the reaction allowed the use of flow cytometry, which gave quantification over three orders of magnitude. An attempt to make the reaction homogenous with fluorescence polarization[127-128] required adapting complex machinery and time-consuming equilibration, and the results generated were not easily quantifiable. To attain mass acceptance by clinical laboratories, a real-time fluorescent assay was needed.

Nadeau et al.[119] have developed a detection probe assay that uses only the enzymes inherent in the reaction, the displacing polymerase, and the restriction enzyme (Figure 4.5B). The probe structure consists of single-stranded DNA (ssDNA) with a restriction enzyme site between the quiescent fluorophores. Either a hairpin loop holds the two labels close together, or the dynamics of a single strand in solution may maintain the quenching. The two labels should not be closer than 9 nucleotides; otherwise they hinder the restriction enzyme. Hairpin probes with a loop of 15 nucleotides maximized sensitivity.

The 3′ end (1 to 30 nucleotides) of the probe is complementary to the target and is extended during the reaction to include a primer-binding site. A primer will bind and make the probe double-stranded, thus producing a cleavage site for the restriction enzyme. The site is designed such that neither strand is protected by the thiolated nucleotide. Cleavage separates the fluorophore and the quencher and releases detectable fluorescence. Because both of the strands are severed if the probe is extended, it cannot act as an amplification primer and does not support amplification. Spurious extension may lead to some loss of efficiency, but not to higher background. Using this method,

ten molecules of a spiked sample were detected in half an hour. This technology has been incorporated in an automated system capable of reliably detecting *Chlamydia trachomatis* or *Neisseria gonorrhoeae* in clinical samples within 1 h.[130]

4.2.4.3 Conclusions

SDA is a very rapid, isothermal amplification reaction. It progresses exponentially until the restriction enzyme concentration becomes limiting.[119] Background DNA competes for restriction enzyme binding and can inflate the amount of expensive enzyme required. The use of a restriction enzyme affects the choice of target because the sequence cannot contain an internal recognition site. Another amplification method has been described that relies on strand displacement and bumper primers but does not require a restriction enzyme. Loop-mediated isothermal amplification (LAMP) generates inverted repeats flanking single-stranded loop sequences available for primer binding. Amplification is extensive but has not been proven with clinical samples.[131,132]

Although target sequence is amplified, the product is not amenable for further manipulation (i.e., cloning, sequencing) because of its heterogeneity. Also, four primers must be optimized for every new reaction. Its utility probably lies in diagnostics, where its speed grants great advantage. Real-time detection allows semiquantitation if a competitor of a known quantity is coamplified.[119,128] Quantitative estimates based on a standard curve were valid over a wide range (500 to 5 million) of original target concentration.

Running this internal standard is a form of multiplexing. In solution, up to three sequences may be amplified together if they use the same primer pairs with specific detection.[133-135] Otherwise, the quadruple primers needed per sequence drastically increase the possibility of cross-reacting with the wrong target or other primers. Multiplexing might be better accomplished on a solid surface. Here, specific primers are electronically placed in discrete zones of amplification, which would allow up to ten or more sequences to be amplified simultaneously.[136,137] Discrimination among four bacterial pathogens and allele-specific detection have been accomplished with this method.[136]

4.2.5 Transcription-Based Amplification

RCA and SDA reflect some aspects of plasmid and circular viral replication. Transcription based-amplification closely mirrors the process of retroviral replication. An RNA template is reverse-transcribed into a double-stranded template of DNA, which is itself transcribed into numerous copies of an RNA template ready to reenter the cycle. The reaction proceeds through numerous continuous cycles of transcription and reverse transcription, catalyzed by RNA polymerase and reverse transcriptase. In an early incarnation of the reaction, the researcher needed to cycle the reaction vessel through a temperature denaturation step to separate the DNA-RNA hybrids,[138] but the current reaction is isothermal and self-perpetuating. The same basic reaction operates under several names: nucleic acid sequence-based amplification (NASBA), transcription-mediated amplification (TMA), and self-sustained sequence replicaton (3SR).

4.2.5.1 Reaction Mechanics

Although double-stranded DNA targets can be amplified with additional denaturation steps (e.g., Reference 139), the reaction works most efficiently with single-stranded RNA targets, heated moderately to destroy the secondary structure. Reverse transcription commences from a complementary downstream primer. The RNA of the newly formed RNA-DNA hybrid is destroyed by RNAse H activity, obviating the need for thermal cycling and leaving a ssDNA capable of binding the upstream primer. The primer is extended through the target sequence and to the 5′ end of the downstream primer, which encodes a promoter sequence for a bacteriophage RNA polymerase.

Electronic Patent Application Fee Transmittal

Application Number:	14067620
Filing Date:	30-Oct-2013
Title of Invention:	Nicking and Extension Amplification Reaction for the Exponential Amplification of Nucleic Acids
First Named Inventor/Applicant Name:	Brian K. Maples
Filer:	Ian J.S. Lodovice/Mary Florczak
Attorney Docket Number:	30171-0025002 / ITI-001

Filed as Large Entity

Filing Fees for Utility under 35 USC 111(a)

Description	Fee Code	Quantity	Amount	Sub-Total in USD($)
Basic Filing:				
Pages:				
Claims:				
Miscellaneous-Filing:				
Petition:				
Patent-Appeals-and-Interference:				
Post-Allowance-and-Post-Issuance:				
Extension-of-Time:				

Description	Fee Code	Quantity	Amount	Sub-Total in USD($)
Extension - 3 months with $0 paid	1253	1	1400	1400
Miscellaneous:				
Submission- Information Disclosure Stmt	1806	1	180	180
		Total in USD ($)		**1580**

Electronic Acknowledgement Receipt

EFS ID:	26621782
Application Number:	14067620
International Application Number:	
Confirmation Number:	4288
Title of Invention:	Nicking and Extension Amplification Reaction for the Exponential Amplification of Nucleic Acids
First Named Inventor/Applicant Name:	Brian K. Maples
Customer Number:	26161
Filer:	Ian J.S. Lodovice/Mary Florczak
Filer Authorized By:	Ian J.S. Lodovice
Attorney Docket Number:	30171-0025002 / ITI-001
Receipt Date:	11-AUG-2016
Filing Date:	30-OCT-2013
Time Stamp:	20:07:51
Application Type:	Utility under 35 USC 111(a)

Payment information:

Submitted with Payment	yes
Payment Type	Deposit Account
Payment was successfully received in RAM	$1580
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IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

First Named Inventor	:	Brian K. Maples	Art Unit	:	1637
Serial No.	:	14/067,620	Examiner	:	Angela Marie Bertagna
Filed	:	October 30, 2013	Conf. No.	:	4288
Title	:	NICKING AND EXTENSION AMPLIFICATION REACTION FOR THE EXPONENTIAL AMPLIFICATION OF NUCLEIC ACIDS			

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Apply any necessary charges or credits to deposit account 06-1050, referencing the above attorney docket number.

Please contact the undersigned if there are any questions regarding this Statement.

Respectfully submitted,

Date: August 11, 2016______________

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Substitute Disclosure Form U.S. Department of Commerce Patent and Trademark Office	Attorney Docket No. 30171-0025002	Application No. 14/067,620
Information Disclosure Statement by Applicant (Use several sheets if necessary)	Applicant Ionian Technologies Inc.	
(37 CFR §1.98(b))	Filing Date October 30, 2013	Group Art Unit 1637

U.S. Patent Documents

Examiner Initial	Desig. ID	Document Number	Publication Date	Patentee	Class	Subclass	Filing Date If Appropriate
	1.	2004/0038256	2/26/2004	Van Ness et al.			
	2.	2004/0058349	3/25/2004	Van Ness et al.			
	3.	5,648,211	7/15/1997	Fraiser et al.			
	4.	6,686,150	2/3/2004	Blackburn et al.			

Foreign Patent Documents or Published Foreign Patent Applications

Examiner Initial	Desig. ID	Document Number	Publication Date	Country or Patent Office	Class	Subclass	Translation Yes	Translation No
	5.							

Other Documents (include Author, Title, Date, and Place of Publication)

Examiner Initial	Desig. ID	Document
	6.	Little et al., "Molecular Diagnostics and Genetics," Clinical Chemistry, 45:6, 777-784 (1999)
	7.	Wang et al., "Homogeneous Real-Time Detection of Single-Nucleotide Polymorphisms by Strand Displacement Amplification on the BD ProbeTec ET System," Clinical Chemistry, 49:10, 1599-1607 (2003)
	8.	C.A. Spargo et al., "Detection of M. tuberculosis DNA using Thermophilic Strand Displacement Amplification," Molecular and Cellular Probes (1996) 10, 247-256
	9.	Chinese Office Action in Application No. 201410465144.4, dated June 28, 2016, pages 1-9
	10.	Notification of Reexamination in Chinese Application No. 200880105424.7, pages 1-8
	11.	Chinese Office Action in Application No. 201410466581.8, dated January 21, 2016, pages 1-20
	12.	Chinese Office Action in Application No. 201410465144.4, dated December 1, 2015, pages 1-7
	13.	Ehses et al., "Optimization and design of oligonucleotide setup for strand displacement amplification," J. Biochem. Biophys. Methods, 63 (2005) 170-186
	14.	Artificial DNA: Methods and Applications (2003), Khudyakov Y.E. & Fields, H.A. (Eds), *Synthetic DNA Used in Amplification Reactions* (pp. 115-159), CRC Press LLC
	15.	Nadezhda V. Zyrina et al., "N.BspD61 DNA nickase strongly stimulates template-independent synthesis of non-palindromic repetitive DNA by Bst DNA polymerase," Biol. Chem. Vol. 388, pp. 367-372, April 2007

Examiner Signature	Date Considered
EXAMINER: Initials citation considered. Draw line through citation if not in conformance and not considered. Include copy of this form with next communication to applicant.	

Substitute Disclosure Form

PTO/SB/06 (09-11)
Approved for use through 1/31/2014. OMB 0651-0032
U.S. Patent and Trademark Office; U.S. DEPARTMENT OF COMMERCE
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PATENT APPLICATION FEE DETERMINATION RECORD Substitute for Form PTO-875	Application or Docket Number 14/067,620	Filing Date 10/30/2013	☐ To be Mailed

ENTITY: ☒ LARGE ☐ SMALL ☐ MICRO

APPLICATION AS FILED – PART I

FOR	NUMBER FILED (Column 1)	NUMBER EXTRA (Column 2)	RATE ($)	FEE ($)
☒ BASIC FEE (37 CFR 1.16(a), (b), or (c))	N/A	N/A	N/A	**280**
☐ SEARCH FEE (37 CFR 1.16(k), (i), or (m))	N/A	N/A	N/A	
☐ EXAMINATION FEE (37 CFR 1.16(o), (p), or (q))	N/A	N/A	N/A	
TOTAL CLAIMS (37 CFR 1.16(i))	minus 20 =	*	X $ =	
INDEPENDENT CLAIMS (37 CFR 1.16(h))	minus 3 =	*	X $ =	
☐ APPLICATION SIZE FEE (37 CFR 1.16(s))	If the specification and drawings exceed 100 sheets of paper, the application size fee due is $310 ($155 for small entity) for each additional 50 sheets or fraction thereof. See 35 U.S.C. 41(a)(1)(G) and 37 CFR 1.16(s).			
☐ MULTIPLE DEPENDENT CLAIM PRESENT (37 CFR 1.16(j))				
* If the difference in column 1 is less than zero, enter "0" in column 2.			TOTAL	**280**

APPLICATION AS AMENDED – PART II

AMENDMENT

08/11/2016	CLAIMS REMAINING AFTER AMENDMENT (Column 1)		HIGHEST NUMBER PREVIOUSLY PAID FOR (Column 2)	PRESENT EXTRA (Column 3)	RATE ($)	ADDITIONAL FEE ($)
Total (37 CFR 1.16(i))	* 36	Minus	** 36	= 0	x $80 =	0
Independent (37 CFR 1.16(h))	* 3	Minus	***3	= 0	x $420 =	0
☐ Application Size Fee (37 CFR 1.16(s))						
☐ FIRST PRESENTATION OF MULTIPLE DEPENDENT CLAIM (37 CFR 1.16(j))						
					TOTAL ADD'L FEE	**0**

AMENDMENT

	CLAIMS REMAINING AFTER AMENDMENT (Column 1)		HIGHEST NUMBER PREVIOUSLY PAID FOR (Column 2)	PRESENT EXTRA (Column 3)	RATE ($)	ADDITIONAL FEE ($)
Total (37 CFR 1.16(i))	*	Minus	**	=	X $ =	
Independent (37 CFR 1.16(h))	*	Minus	***	=	X $ =	
☐ Application Size Fee (37 CFR 1.16(s))						
☐ FIRST PRESENTATION OF MULTIPLE DEPENDENT CLAIM (37 CFR 1.16(j))						
					TOTAL ADD'L FEE	

* If the entry in column 1 is less than the entry in column 2, write "0" in column 3.
** If the "Highest Number Previously Paid For" IN THIS SPACE is less than 20, enter "20".
*** If the "Highest Number Previously Paid For" IN THIS SPACE is less than 3, enter "3".
The "Highest Number Previously Paid For" (Total or Independent) is the highest number found in the appropriate box in column 1.

LIE
GLORIA J. TRAMMELL

This collection of information is required by 37 CFR 1.16. The information is required to obtain or retain a benefit by the public which is to file (and by the USPTO to process) an application. Confidentiality is governed by 35 U.S.C. 122 and 37 CFR 1.14. This collection is estimated to take 12 minutes to complete, including gathering, preparing, and submitting the completed application form to the USPTO. Time will vary depending upon the individual case. Any comments on the amount of time you require to complete this form and/or suggestions for reducing this burden, should be sent to the Chief Information Officer, U.S. Patent and Trademark Office, U.S. Department of Commerce, P.O. Box 1450, Alexandria, VA 22313-1450. DO NOT SEND FEES OR COMPLETED FORMS TO THIS ADDRESS. **SEND TO: Commissioner for Patents, P.O. Box 1450, Alexandria, VA 22313-1450.**

If you need assistance in completing the form, call 1-800-PTO-9199 and select option 2.

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

First Named Inventor	:	Brian K. Maples	Art Unit	:	1637
Serial No.	:	14/067,620	Examiner	:	Angela Marie Bertagna
Filed	:	October 30, 2013	Conf. No.	:	4288
Title	:	NICKING AND EXTENSION AMPLIFICATION REACTION FOR THE EXPONENTIAL AMPLIFICATION OF NUCLEIC ACIDS			

MAIL STOP AMENDMENT
Commissioner for Patents
P.O. Box 1450
Alexandria, VA 22313-1450

INFORMATION DISCLOSURE STATEMENT

Please consider the references listed on the enclosed PTO-SB-08 or Disclosure Form. Foreign patent documents and non-patent literature are enclosed; cited U.S. patents and patent application publications will be provided on request. A copy of a communication from a foreign patent office in a counterpart application is also enclosed.

This statement is being filed after a first action on the merits, but before receipt of a final action or a notice of allowance. Each item of information contained in this information disclosure statement was first cited in any communication from a foreign patent office in a counterpart foreign application not more than three months prior to the filing of this information disclosure statement. See 37 CFR 1.97(e)(1).

Apply any necessary charges or credits to deposit account 06-1050, referencing the above attorney docket number.

Please contact the undersigned if there are any questions regarding this Statement.

Respectfully submitted,

Date: May 18, 2016

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U.S. Patent Documents							
Examiner Initial	Desig. ID	Document Number	Publication Date	Patentee	Class	Subclass	Filing Date If Appropriate
	1.	6,403,341	06/11/2002	Barnes et al.			
	2.	6,566,103	05/20/2003	Wijnhoven et al.			
	3.	2008/038782	02/14/2008	Borns			

Foreign Patent Documents or Published Foreign Patent Applications								
Examiner Initial	Desig. ID	Document Number	Publication Date	Country or Patent Office	Class	Subclass	Translation Yes	Translation No
	4.	WO2015/113828	08/06/2015	WIPO				
	5.	WO2016/004333	01/07/2016	WIPO				
	6.	WO2003/012066	02/13/2003	WIPO				
	7.	WO2007/096182	08/30/2007	WIPO				
	8.	GB 2416352	01/25/2006	United Kingdom				
	9.	WO2011/085160	07/14/2011	WIPO				
	10.	EP 2824189	01/14/2015	Europe				

Other Documents (include Author, Title, Date, and Place of Publication)		
Examiner Initial	Desig. ID	Document
	11.	Extended European Search Report in corresponding Application No. 13799829.0, dated March 31, 2016, pages 1-10
	12.	Arena et al., "Calcium- And Magnesium-EDTA Complexes. Stability Constants and Their Dependence on Temperature and Ionic Strength," Thermochimica Acta, 61 (1983) 129-138
	13.	Examiner's Report in corresponding Canadian Application No. 2,693,805 dated March 18, 2016, pages 1-5

Examiner Signature	Date Considered
EXAMINER: Initials citation considered. Draw line through citation if not in conformance and not considered. Include copy of this form with next communication to applicant.	

Substitute Disclosure Form

(12) INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(19) World Intellectual Property Organization
International Bureau

(43) International Publication Date
6 August 2015 (06.08.2015)

(10) International Publication Number
WO 2015/113828 A1

(51) **International Patent Classification**:
C12Q 1/68 (2006.01)

(21) **International Application Number**:
PCT/EP2015/050671

(22) **International Filing Date**:
15 January 2015 (15.01.2015)

(25) **Filing Language**: English

(26) **Publication Language**: English

(30) **Priority Data**:
14153386.9 31 January 2014 (31.01.2014) EP

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(81) **Designated States** *(unless otherwise indicated, for every kind of national protection available)*: AE, AG, AL, AM, AO, AT, AU, AZ, BA, BB, BG, BH, BN, BR, BW, BY, BZ, CA, CH, CL, CN, CO, CR, CU, CZ, DE, DK, DM, DO, DZ, EC, EE, EG, ES, FI, GB, GD, GE, GH, GM, GT, HN, HR, HU, ID, IL, IN, IR, IS, JP, KE, KG, KN, KP, KR, KZ, LA, LC, LK, LR, LS, LU, LY, MA, MD, ME, MG, MK, MN, MW, MX, MY, MZ, NA, NG, NI, NO, NZ, OM, PA, PE, PG, PH, PL, PT, QA, RO, RS, RU, RW, SA, SC, SD, SE, SG, SK, SL, SM, ST, SV, SY, TH, TJ, TM, TN, TR, TT, TZ, UA, UG, US, UZ, VC, VN, ZA, ZM, ZW.

(84) **Designated States** *(unless otherwise indicated, for every kind of regional protection available)*: ARIPO (BW, GH, GM, KE, LR, LS, MW, MZ, NA, RW, SD, SL, ST, SZ, TZ, UG, ZM, ZW), Eurasian (AM, AZ, BY, KG, KZ, RU, TJ, TM), European (AL, AT, BE, BG, CH, CY, CZ, DE, DK, EE, ES, FI, FR, GB, GR, HR, HU, IE, IS, IT, LT, LU, LV, MC, MK, MT, NL, NO, PL, PT, RO, RS, SE, SI, SK, SM, TR), OAPI (BF, BJ, CF, CG, CI, CM, GA, GN, GQ, GW, KM, ML, MR, NE, SN, TD, TG).

Published:
— *with international search report (Art. 21(3))*

WO 2015/113828 A1

(54) **Title**: CATION CHELATOR HOT START

(57) **Abstract**: The invention is in the field of regulation of enzymatic activity in nucleic acid modifying reactions. It describes a method of regulating enzymatic activity by adding chelating agents to the reaction composition and exploits the fact that both the binding of divalent cations to these chelating agents and the pH of commonly used buffers is temperature dependent. PCR experiments that are hampered by non-specific side products can be regulated such that the target sequence is amplified in a more specific manner.

CATION CHELATOR HOT START

Field of the invention

The present invention relates to the field of nucleic acid chemistry. More particular, it relates to the regulation of enzyme activity in the field of nucleic acid modifying reactions.

Background

Nucleic acid modifying reactions play a pivotal role in modern biological and pharmaceutical research, both in the academic and industrial settings. Such reactions cover a wide range of applications, ranging from nucleic acid amplification reactions to regulated and specific cleavage of nucleic acids. These are mediated by enzymes that have been studied extensively for the past decades.

Amplification of target nucleic acid sequences is of importance to modern biological and pharmaceutical industry. Large-scale robotic facilities used in industrial research depend on the accurate and efficient regulation of amplification conditions to ensure that the target sequences are correctly amplified for downstream applications.

Regulation of the activity of such enzymes is however not a trivial task. In the case of polymerases, efficient amplification is dependent on a complex interplay of parameters such as primer length, GC content of both primer and target sequences as well as ionic strength and composition of the reaction buffer. Further, non-specific binding of primers is often observed at lower temperatures during the amplification cycles. This increases the fraction of non-specific side products and lowers the overall efficiency of the amplification reaction.

To address this, recent developments in the field of polymerases describe “Hot Start” polymerases. This class of enzymes is either chemically inactivated or has the active site blocked due to binding of a specific antibody or an aptamer. After an activation step at high temperature, the chemical modification is cleaved off and the enzyme is activated.

In addition to “Hot Start” polymerases, so called “Hot Start” primers and “Hot Start” nucleotides have also been developed. These are chemically modified primers, wherein the modification is cleaved off at high temperatures and thus the primer is rendered functional and is able to hybridize to its target sequence. However, synthesis of such primers is expensive and requires more time than standard primers. Both in the case of the “Hot Start” primer and polymerases, the blocking features are only available once since the heat-induced removal of the chemical modification is irreversible.
There is a need for methods that allow for the regulation of enzymatic activity without elaborate modification of enzymes and substrates.

Brief description of the invention

The present invention relates to a method of regulation of enzymatic activity by controlling the concentration of divalent cations in the reaction composition.

The reaction composition comprises at least one enzyme, wherein the activity of said enzyme is dependent on divalent cations; a chelating agent; a divalent cation, wherein the binding of said cation to said chelating agent is dependent on pH and/or temperature of the reaction composition; a buffering system, wherein the acid dissociation constant is temperature dependent, such that a change in temperature results in a change of pH of the aqueous solution; and a substrate of said enzyme. In addition, changing the temperature in the reaction composition results in divalent cations which are bound to chelating agents being released from these complexes and thereby the enzyme is activated or increases activity. Chelating said cation does not have structural consequences; selectively complexing said cation modulates activity. The change in activity is reversible; inactivation by chelating can be reversed by releasing said cation upon temperature increase.

The invention also relates to a kit for performing a nucleic acid modifying reaction and comprises a buffer system, a chelating agent, a nucleic acid modifying enzyme and a divalent cation for said enzyme.

Detailed description of the invention

The present invention relates to a method for the regulation of enzyme activity in a reaction composition. The reaction composition comprises at least one enzyme, wherein
5 the activity of said enzyme is dependent on divalent cations; a chelating agent; a divalent cation, wherein the binding of said cation to said chelating agent is dependent on pH and/or temperature of the reaction composition; a buffering system, wherein the acid dissociation constant is temperature dependent, such that a change in temperature results in a change of pH of the aqueous solution; and a substrate of said enzyme. In
10 addition, changing the temperature in the reaction composition results in divalent cations which are bound to chelating agents being released from these complexes and thereby the enzyme is activated or increases activity. Chelating said cation does not have structural consequences; selectively complexing said cation modulates activity. The change in activity is reversible; inactivation by chelating can be reversed by releasing said
15 cation upon temperature increase.

In a preferred embodiment, the present invention relates to a method for the regulation of enzyme activity in a reaction composition. The reaction composition comprises at least one enzyme, wherein the activity of said enzyme is dependent on divalent cations; a
20 chelating agent; a divalent cation, wherein the binding of said cation to said chelating agent is dependent on pH of the reaction composition; a buffering system, wherein the acid dissociation constant is temperature dependent, such that a change in temperature results in a change of pH of the aqueous solution; and a substrate of said enzyme. In addition, changing the temperature in the reaction composition results in divalent cations
25 which are bound to chelating agents being released from these complexes and thereby the enzyme is activated or increases activity. Chelating said cation does not have structural consequences; selectively complexing said cation modulates activity. The change in activity is reversible; inactivation by chelating can be reversed by releasing said cation upon temperature increase.

30

In one embodiment of the invention the enzyme is a nucleic acid modifying enzyme.

In one embodiment, the activity of the nucleic acid modifying enzyme comprises substrate binding and substrate processing activity.

In a preferred embodiment the nucleic acid modifying enzyme is selected from the group of polymerases, transcriptases and cation-dependent nucleases.

In a more preferred embodiment the polymerase is selected from the group of organisms comprising *Thermus, Aquifex*, *Thermotoga*, *Thermocridis*, *Hydrogenobacter*, *Thermosynchecoccus, Thermoanaerobacter, Pyrococcales, Thermococcus, Bacilus, Sulfolobus* and non-thermophiles. Preferably the viral reverse transcriptases are from MMLV, AMV HIV, EIAV and/or the nuclease is a bovine DNase.

In the most preferred embodiment the polymerase is selected from the group of organisms comprising *Aquifex aeolicus*, *Aquifex pyogenes*, *Thermus thermophilus*, *Thermus aquaticus*, *Thermotoga neopolitana*, *Thermus pacificus*, *Thermus eggertssonii* and *Thermotoga maritima*.

In particular, the invention also describes a method, wherein the removal of said divalent cation results in decreased or loss of activity of said nucleic acid modifying enzyme.

This represents an option to regulate enzymatic activity and substrate binding is at the level of the concentration of divalent cations in the reaction composition. For instance, in the case of polymerases and many nucleases, the concentration of divalent ions such as magnesium, calcium and others is crucial to the activity of the enzyme. A reduced level of said cations leads to vastly decreased activity or even abolishes enzymatic activity. In the case of polymerases, stability of hybridization of the primers to the target sequence is greatly reduced. Many nucleases possess a divalent cation in the active site that is crucial to substrate processing. A way to regulate enzymatic activity on the level of ion concentration exploits the fact that both the pH value of buffers routinely used in enzymatic reaction mixtures and the ability of chelating agents to bind ions is temperature-dependent. In addition to polymerases, other nucleic acid modifying

enzymes such as nucleases also depend on divalent ions in their active site and therefore can be regulated as described above.

The invention also relates to a method, wherein the activity of said nucleic acid modifying 5 enzyme is selected from the group comprising amplification, reverse transcription, isothermal amplification, sequencing and hydrolytic cleavage of ester bonds, preferably amplification, reverse transcription, and hydrolytic cleavage of ester bonds.

In a preferred embodiment of the invention the chelating agent is selected from the 10 group comprising ethylene di amine tetra acetic acid (EDTA), ethylene glycol bis(amino ethyl) N, N'-tetra acetic acid and nitrile acetic acid (NTA). Particularly preferred is EGTA.

In one embodiment the divalent cation is selected from the group comprising Mg^{2+}, Ca^{2+}, Mn^{2+}, Cu^{2+}, Fe^{2+}, Ni^{2+}, Zn^{2+} and Co^{2+}. In a preferred embodiment, the chelating agent is 15 EDTA and the cations are selected from Mg^{2+}, Ca^{2+}, Mn^{2+}, Cu^{2+}, Ni^{2+}, Zn^{2+} and/or Co^{2+}.

In one embodiment, several cations selected from the group comprising Mg^{2+}, Ca^{2+}, Mn^{2+}, Cu^{2+}, Fe^{2+}, Ni^{2+}, Zn^{2+} and Co^{2+} are present in the reaction.

20 In one embodiment the chelating agent is EGTA and the cations are Ca^{2+} and/or Mg^{2+}.

In one embodiment the chelating agent is NTA and the cations are $Ca^{2+}$and/or Cu^{2+} and/or Co^{2+}.

In another embodiment of the invention the buffer is suitable for enzymatic reactions. Preferably the buffer is selected from Table 4. Tris buffer is used in enzymatic reactions, 25 preferably in PCR experiments. The pH value of Tris buffer is temperature dependent. At room temperature, the pH is around 8.7. A shift in pH of 0.03 pH units per °C is observed. Therefore, at 95 °C the pH is 6.6.

In one embodiment, the concentration of the buffer system is between 0.01 and 100 mM, 30 preferably between 0.1 and 50 mM, more preferably between 1 and 30 mM and most preferably between 5 and 15 mM.

In one embodiment, the concentration of the divalent cation in the reaction is between 0.01 and 20 mM, preferably between 0.1 and 10 mM, most preferable between 1 and 8 mM.

In one embodiment, the concentration of the chelating agent is between 0.05 and 50 mM, more preferably between 0.1 and 20 mM, even more preferably between 0.5 and 10 mM, and most preferably between 1 and 8 mM.

In one embodiment, the pH varies during the reaction in response to the temperature change by at least 0.05 pH units, preferably by at least 0.1, more preferably by at least 0.5, even more preferably by at least 1 and most preferably by at least 2 pH units.

The invention relates to a method, wherein the reaction composition comprises a buffer system, preferably a Tris buffer system, wherein the divalent cation is Mg^{2+}, preferably at a concentration between 0.01 and 20 mM; wherein the chelating agent is EGTA at a concentration between 0.05 and 50 mM and wherein the nucleic acid modifying enzyme is a DNA polymerase, preferably a hot start polymerase. Preferred EGTA concentration is between 0.1 mM and 20 mM, more preferred between 0.5 mM and 10 mM.

The invention also relates to a method, wherein the reaction composition comprises a buffer system, preferably a Tris buffer system; wherein the divalent cation is selected from the group of Mg^{2+}, Ca^{2+}, Mn^{2+}, Cu^{2+}, Fe^{2+}, Ni^{2+}, Zn^{2+} and Co^{2+}; wherein the chelating agent is selected from the group of EGTA, EDTA and NTA and wherein the nucleic acid enzyme is a nuclease.

Further, the invention relates to a kit for performing a nucleic acid modifying reaction comprising a buffer system, a chelating agent, a nucleic acid modifying enzyme and a divalent cation for said enzyme.

Examples

Selection of chelating agent

5 Tris buffer is routinely used in PCR buffers. At room temperature the pH of a Tris based PCR buffer is 8.7. Tris shows a temperature-dependent shift in pH value of 0.03 pH units per °C. This means that at 95 °C the pH value is 6.6. In order to select a chelating agent for PCR experiments, the pH-dependency of the binding constants of three different chelating agents, NTA; EDTA and EGTA, was investigated. Known pK values from literature
10 for every chelating agent were used to determine the pH dependency of the complex formation (Figure 1).The correlation curve for EGTA shows a strong correlation between pH value and binding constant. Therefore, EGTA was selected for subsequent experiments.

15 **Endpoint PCR**

An amplification experiment was performed using a test system that is known to be prone to produce non-specific side products. A genomic DNA sequence of 1.2 kb was the target sequence. Primers HugA and HugB were used.
20 The primer sequences are as follows:

SEQ ID NO	Primer name	Sequence
1	HugA	CACACAGCGATGGCAGCTATGC
2	HugB	CCCAGTGATGGGCCAGCT

Table 1: Primer sequences used for endpoint PCR experiment.

Reactions with and without EGTA were performed in parallel. In set A, the magnesium
25 concentration was varied in 1 mM steps, start and end point were 5 and 10 mM respectively. In set B, the start point was 0.5 mM and the end point was 4 mM Mg. The setup is described in Table 2.

	MM A	MM B	
Puffer (-Mg)	1	1	X
Taq	0.625	0.625	Units
Primer HugA	0.5	0.5	μM
Primer HugB	0.5	0.5	μM
dNTPs	0.2	0.2	mM
gDNA	10	10	ng
EGTA	5	0	mM

Table 2: HugI PCR reactions mixture.

The amplification program was as follows (Table 3):

Time [min:sec]	**Temp [°C]**
03:00	95
00:30	94
01:00	59
01:00	72
10:00	72
	4

Table 3: Amplification program of HugI PCR.

5

35 cycles were performed.

The analysis of the PCR reactions on the agarose gel (Figure 2) shows that the reactions containing EGTA as a chelating agent are more specific as of having less side products compared to those reactions without EGTA. Other buffers routinely used in enzymatic

10 reactions are listed in Table 4.

Buffer ID No.	Product #	Description	Useful pH Range	CAS Number	pKa
1	A3594	ACES BioPerformance Certified, ≥99.0%	6.1 - 7.5	7365-82-4	6.80
2	B4554	BES BioPerformance Certified, cell culture tested, ≥99.0%	6.4 - 7.8	10191-18-1	7.10
3	B4429	BIS-TRIS BioPerformance Certified, cell culture tested, suitable for insect cell culture, ≥98%	5.8 - 7.2	6976-37-0	6.50

4	B4679	BIS-TRIS propane BioPerformance Certified, cell culture tested, ≥99.0%	6.3 - 9.5	64431-96-5	6.80
5	E0276	EPPS BioPerformance Certified, cell culture tested, ≥99.5% (titration)	7.3 - 8.7	16052-06-5	8.00
6	G3915	Gly-Gly BioPerformance Certified, cell culture tested, ≥99%	7.5 - 8.9	556-50-3	8.20
7	H4034	HEPES BioPerformance Certified, ≥99.5% (titration), cell culture tested	6.8 - 8.2	7365-45-9	7.50
8	H3784	HEPES sodium salt BioPerformance Certified, suitable for cell culture, ≥99.5%	6.8 - 8.2	75277-39-3	7.50
9	H3662	HEPES sodium salt solution 1M, BioReagent, suitable for cell culture		75277-39-3	
10	H3537	HEPES solution 1 M, BioReagent, suitable for cell culture, suitable for molecular biology, 0.2 µm filtered	6.8 - 8.2	7365-45-9	
11	M2933	MES hydrate BioPerformance Certified, suitable for cell culture, ≥99.5%	5.5 - 6.7	4432-31-9 (anhydrous)	6.10
12	M3058	MES sodium salt BioPerformance Certified, suitable for cell culture	5.5 - 6.7	71119-23-8	6.10
13	M1317	MES solution 1 M, BioReagent, for molecular biology, suitable for cell culture	5.5 - 6.7		
14	M3183	MOPS BioPerformance Certified, cell culture tested, ≥99.5% (titration)	6.5 - 7.9	1132-61-2	7.20
15	M9024	MOPS sodium salt BioPerformance Certified, suitable for cell culture, ≥99.5%	6.5 - 7.9	71119-22-7	7.20
16	M1442	MOPS solution BioReagent, 1 M, for molecular biology, suitable for cell culture	6.5 - 7.9		
17	P1851	PIPES BioPerformance Certified, suitable for cell culture, ≥99%	6.1 - 7.5	5625-37-6	6.80
18	P5493	Phosphate buffered saline 10× concentrate, BioPerformance Certified, suitable for cell culture			
19	P5368	Phosphate buffered saline BioPerformance Certified, pH 7.4			
20	S6191	Sodium chloride BioPerformance Certified, ≥99.5% (titration), Cell Culture Tested		7647-14-5	
21	S6566	Sodium phosphate monobasic Biotechnology Performance Certified, Cell Culture Tested		7558-80-7	
22	T5316	TAPS BioPerformance Certified, cell culture tested, ≥99.5% (titration)	7.7 - 9.1	29915-38-6	8.40
23	T5441	TAPS sodium salt BioPerformance Certified, suitable for cell culture, ≥99%	7.7 - 9.1	91000-53-2	8.40
24	T5691	TES BioPerformance Certified, cell culture tested, ≥99% (titration)	6.8 - 8.2	7365-44-8	7.50

25	T5816	Tricine BioPerformance Certified, cell culture tested, ≥99% (titration)	7.4 - 8.8	1389475.00	8.10
26	T7193	Trizma® Pre-set crystals BioPerformance Certified, pH 7.0, average Mw 154.8	7.0 - 9.0		
27	T9943	Trizma® Pre-set crystals BioPerformance Certified, pH 7.0, average Mw 154.8	7.0 - 9.0		
28	T7443	Trizma® Pre-set crystals BioPerformance Certified, pH 7.2, average Mw 153.8	7.0 - 9.0		
29	T7693	Trizma® Pre-set crystals BioPerformance Certified, pH 7.4, average Mw 151.6	7.0 - 9.0		
30	T0319	Trizma® Pre-set crystals BioPerformance Certified, pH 7.4, average Mw 151.6	7.0 - 9.0		
31	T7818	Trizma® Pre-set crystals BioPerformance Certified, pH 7.5, average Mw 150.6	7.0 - 9.0		
32	T7943	Trizma® Pre-set crystals BioPerformance Certified, pH 7.6, average Mw 149.0	7.0 - 9.0		
33	T8068	Trizma® Pre-set crystals BioPerformance Certified, pH 7.7, average Mw 147.6	7.0 - 9.0		
34	T8193	Trizma® Pre-set crystals BioPerformance Certified, pH 7.8, average Mw 145.8	7.0 - 9.0		
35	T8443	Trizma® Pre-set crystals BioPerformance Certified, pH 8.0, average Mw 141.8	7.0 - 9.0		
36	T0819	Trizma® Pre-set crystals BioPerformance Certified, pH 8.0, average Mw 141.8	7.0 - 9.0		
37	T8568	Trizma® Pre-set crystals BioPerformance Certified, pH 8.1, average Mw 139.8	7.0 - 9.0		
38	T8943	Trizma® Pre-set crystals BioPerformance Certified, pH 8.3, average Mw 135.4	7.0 - 9.0		
39	T8818	Trizma® Pre-set crystals BioPerformance Certified, pH 8.5, average Mw 131.4	7.0 - 9.0		
40	T1194	Trizma® Pre-set crystals BioPerformance Certified, pH 8.5, average Mw 131.4	7.0 - 9.0		
41	T9443	Trizma® Pre-set crystals BioPerformance Certified, pH 8.8, average Mw 127.2	7.0 - 9.0		
42	T9568	Trizma® Pre-set crystals BioPerformance Certified, pH 8.9, average Mw 125.6	7.0 - 9.0		
43	T9693	Trizma® Pre-set crystals BioPerformance Certified, pH 9.0, average Mw 124.6	7.0 - 9.0		
44	T1444	Trizma® Pre-set crystals BioPerformance Certified, pH 9.0, average Mw 124.6	7.0 - 9.0		
45	T9818	Trizma® Pre-set crystals BioPerformance Certified, pH 9.1, average Mw 123.0	7.0 - 9.0		
46	T0194	Trizma® Pre-set crystals pH 7.2, average Mw 153.8	7.0 - 9.0		
47	T0444	Trizma® Pre-set crystals pH 7.5, average	7.0 - 9.0		

		Mw 150.6			
48	T0569	Trizma® Pre-set crystals pH 7.6, average Mw 149.0	7.0 - 9.0		
49	T0694	Trizma® Pre-set crystals pH 7.7, average Mw 147.6	7.0 - 9.0		
50	T0944	Trizma® Pre-set crystals pH 8.1, average Mw 139.8	7.0 - 9.0		
51	T1069	Trizma® Pre-set crystals pH 8.3, average Mw 135.4	7.0 - 9.0		
52	T1319	Trizma® Pre-set crystals pH 8.8, average Mw 127.2	7.0 - 9.0		
53	T6066	Trizma® base BioPerformance Certified, meets EP, USP testing specifications, cell culture tested, ≥99.9% (titration)	41524.00	77-86-1	8.10
54	T5941	Trizma® hydrochloride BioPerformance Certified, cell culture tested, ≥99.0% (titration)	7.0 - 9.0	1185-53-1	8.10
55	T1819	Trizma® hydrochloride solution pH 7.0, 1 M, BioReagent, for molecular biology, suitable for cell culture	7.0 - 9.0		
56	T2069	Trizma® hydrochloride solution pH 7.2, 1 M, BioReagent, for molecular biology, suitable for cell culture	7.0 - 9.0		
57	T1944	Trizma® hydrochloride solution pH 7.4, 0.1 M	7.0 - 9.0		
58	T2194	Trizma® hydrochloride solution pH 7.4, 1 M, BioReagent, for molecular biology, suitable for cell culture	7.0 - 9.0		
59	T2319	Trizma® hydrochloride solution pH 7.5, 1 M, BioReagent, for molecular biology, suitable for cell culture	7.0 - 9.0		
60	T2944	Trizma® hydrochloride solution pH 7.5, 2 M, BioReagent, for molecular biology, suitable for cell culture	7.0 - 9.0		
61	T2444	Trizma® hydrochloride solution pH 7.6, 1 M, BioReagent, for molecular biology, suitable for cell culture	7.0 - 9.0		
62	T2569	Trizma® hydrochloride solution pH 7.8, 1 M, BioReagent, for molecular biology, suitable for cell culture	7.0 - 9.0		
63	T2694	Trizma® hydrochloride solution pH 8.0, 1 M, BioReagent, for molecular biology, suitable for cell culture	7.0 - 9.0		
64	T3069	Trizma® hydrochloride solution pH 8.0, 2 M, BioReagent, for molecular biology, suitable for cell culture	7.0 - 9.0		

65	T2819	Trizma® hydrochloride solution pH 9.0, 1 M, BioReagent, for molecular biology, suitable for cell culture	7.0 - 9.0		

Table 4: List of buffers commonly used in enzymatic reactions.

Modulation of residual activity of chemically inactivated *Taq* DNA polymerase using EGTA/EDTA

5 The following experiments employed a system to detect the formation of primer dimers in a PCR reaction mixture using residual active *Taq* polymerase molecules. Herein, bisulphite-treated DNA is used as a template. As a consequence of the bisulphite treatment, which entails the chemical modification of non-methylated cytosines to uracil), said template only consists of three bases. Since bisulphite treatment only works when using single stranded DNA, the majority of DNA after 10 completion of said bisulphite treatment is single stranded. Primers that are used for amplification of such DNA sequences are characterized by reduced complexity since they only consist of three bases. Hence these primers are prone to dimer formation and are very likely to be able to bind > 100.000 times to said bisulphite-treated DNA.

15 Genomic DNA was propagated using the Qiagen REPLI g Midi Kit according to the manufacturer's protocol. Subsequently, 1 µg of said genomic DNA was used in 10 independent reactions wherein the DNA was subjected to bisulphite treatment using the EpiTect Bisulfite Kit followed by purification. The resulting DNA of each reaction was pooled and used in the subsequent amplification reactions. Primer sequences are shown in Table 5.

20

SEQ ID NO	Primer NO	Sequence
3	1	ACCCCCACTAAACATACCCTTATTCT
4	2	GGGAGGGTAATGAAGTTGAGTTTAGG

Table 5: Primers used in amplification of bisulphite-treated DNA.

Reagent	Concentration	Volume (µl)
EpiTect HRM PCR Kit	2x	12.5
Primer 1	10 µM	1.875
Primer 2	10 µM	1.875
Bisulphite-treated DNA	10 ng/µl	1
Water or EGTA	1-x mM	5

Water		2.75

Table 6: Amplification reaction mixture.

The final EGTA concentration was between 0.25 and 10 mM.

5 One set of samples consisting of two reactions was incubated on ice for 120 min, whereas the other set of samples also consisting of two reactions was incubated at room temperature for 120 min. Subsequently both sets of samples were analyzed using the Rotor-Gene Q 5plex HRM System. The cycling program is shown in Table 7.

95°C - 5'	
95°C - 10"	
55°C - 30"	x 40
72°C - 10"	
HRM 68°C-82°C	

10

Table 7: Cycling program used in the amplification of bisulphite-treated DNA.

Ct values are summarized in Table 8 and Figure 3 shows the respective melting curves.

		Room temperature			**On ice**	
EGTA [mM]	Ct	Average	Standard deviaton	Ct	Average	Standard deviation
-	25,01	24,92	0,1	25,29	25,28	0,01
	24,82			25,26		
0,25	25,2	25,31	0,11	25,34	25,43	0,09
	25,42			25,51		
0,5	25,55	25,51	0,04	25,53	25,58	0,04
	25,47			25,62		
0,75	25,91	25,86	0,05	25,78	25,83	0,05
	25,8			25,88		
1	26,47	26,49	0,02	26,59	26,55	0,04
	26,5			26,61		
1,5	27,76	27,82	0,06	28,17	28,19	0,02
	27,88			28,21		
2	29,9	29,81	0,09	30,16	29,35	0,82
	29,71			28,53		
4	37,32	37,13	0,1	38,37	38,26	0,11
	37,02			38,14		
8						
10	35,18	34,59	0,59			
	34					

15

Table 8: Summary of results obtained from the amplification experiment of bisulphite-treated DNA in the presence of different EGTA concentrations.

Samples that had been incubated on ice without the addition of EGTA showed a Ct value of 25.28 and a specific melting curve (Figure 3A) whereas the Ct value in the case of the samples that had been incubated at room temperate is shifted to the right (24.92). In this case no specific product was observed. Addition of EGTA up to a final concentration of 0.75 mM resulted in an increase of specificity without affecting the Ct values. The amount of specific product increased rapidly. In the case of EGTA concentrations exceeded 2 mM, successful amplification was prevented (Figure 3H).

In the follow-up experiment said primers and said bisulphite-treated DNA were used in amplification reactions wherein the magnesium dependency was analyzed. The composition of the reaction mixtures is shown in Table 9.

Reagent	Concentration	Volume (µl)
EpiTect HRM PCR Kit	2x	12.5
Primer 1	10 µM	1.875
Primer 2	10 µM	1.875
Bisulphite-treated DNA	10 ng/µl	1
Water or EGTA	5 mM	2.5
Magnesium	0.1 mM-0.6 mM	2.5
Water		2.75

Table 9: Amplification reaction mixture.

The final concentration of EGTA was 5 mM. The HRM master mix was supplemented with 0.1 0.6 mM magnesium.

Two sets of reactions consisting of duplicates were used in the amplification experiment. One set of samples was incubated on ice for 120 min, whereas the other set of samples was incubated at room temperature for 120 min. Subsequently the samples were analyzed using the Rotor-Gene Q 5plex HRM System. The cycling program was the same as shown in Table 6. The results are shown in Table 9 and Figure 4. Ct values corresponding to the samples incubated at room temperature and on ice respectively are shown in Table 10. Figure 4 shows the respective melting curves.

			Room temperature			On ice		
DNA	EGTA	$MgCl_2$ [mM]	Ct	Average	Standard deviation	Ct	Average	Standard deviation
	-	-	22,41	22,62	0,21	25,32	25,46	0,14
			22,82			25,59		
		0 mM	28,86	28,77	0,09	28,74	28,82	0,08
			28,67			28,89		
		0,1	27,53	27,62	0,09	27,82	27,81	0,02
			27,71			27,79		
10 ng	5 mM	0,2	26,69	26,98	0,29	26,85	27,12	0,27
			27,27			27,39		
		0,3	26,14	26,39	0,25	26,25	26,38	0,13
			26,64			26,51		
		0,4	26,06	26,15	0,09	26,01	26,08	0,06
			26,24			26,14		
		0,5	25,82	25,84	0,02	25,81	25,85	0,04
			25,86			25,89		
		0,6	25,55	25,52	0,03	25,61	25,59	0,03
			25,49			25,56		

Table 10: Summary of results obtained from the amplification experiment of bisulphite-treated DNA in the presence of EGTA and different magnesium concentrations.

The experiment showed that in the case of the samples incubated on ice without the addition of EGTA a Ct value of 25.46 and a specific melting curve was obtained, whereas incubation at room temperature resulted in a shift of the Ct value(22.62) and no specific amplification product was observed. Addition of EGTA up to a final concentration of 5 mM led to increased specificity. The Ct value when using 5 mM EGTA was 28.77 and 28.82 respectively. Increasing the magnesium concentration resulted in lower Ct values whilst maintaining specificity.

Modulation of DNase activity

In this set of experiments means of modulating acitivity of DNase, a nuclease isolated from bovine pancreas, were investigated.

Human genomic DNA was propagated using the REPLI g Midi Kit (Qiagen) according to the manufacturer's instructions. DNase activity was analyzed in 10 µl reactions. Each reaction contained 50 mM Tris pH 8.2 as the reaction buffer, ~ 1µg genomic DNA, 1 mM $MgCl_2$, and 50 µM $CaCl_2$. Three different amounts of DNase (0.01, 0.1 and 1 U) were used. The samples were incubated at two different temperatures, 42 °C and on ice, for 5 and 15 min respectively. DNA degradation was terminated by adding EDTA to a final concentration of 8.33 mM and samples were incubated on ice prior to analysis of the reaction products using a 0.5 % agarose gel.
The results are shown in Figure 5. The gel shows that DNase is active on ice. Reaction time and the amount of DNase strongly influence completeness of enzymatic digestion. Incubation of said

genomic DNA on ice for 15 min using 1 U DNase led to complete degradation of the sample (lane 3). Incubation at 42 °C led to complete degradation when using any of the amounts of enzyme already after 5 min (lanes 8-13, '42 °C').

5 Addition of EGTA to a final concentration of 100 µM led to almost complete inhibition of degradation for any of the amounts of DNase that were used (lanes 2-7, 'on ice' '100 µM EGTA'). Exempt from this is the reaction using 1 U DNase for 15 min (lane 3 'on ice' '100 µM EGTA'). However, in this case degradation is significantly reduced compared to the sample without EGTA. Increasing the temperature to 42 °C largely restored DNase activity (lanes 8-13 '42 °C' '100 µM 10 EGTA').

In the follow-up experiment EDTA was used as a chelating agent. The procedure of genomic DNA propagation as well the buffer and reaction conditions were equivalent to the experiment as described above.

15

The reaction products were analyzed using a 0.5 % agarose gel (Figure 6). Lanes 1-6 correspond to samples where the reaction was performed on ice. A comparison of lanes 1-3 and lanes 4-6 shows that addition of EDTA largely reduced enzymatic activity. An increase in reaction temperature to 42 °C results in bound Ca^{2+} ions being released from complexes with EDTA and thereby restoring 20 enzymatic activity. Complete DNA degradation can be observed in lanes 7-12.

In summary, both examples show that chelating agents can be used to inhibit DNase activity and that shifting the reaction temperature restores enzymatic activity, thereby validating said system of activity regulation.

25

Figure captions

Figure 1: Selection of chelating agents. pH-dependency of pK values of EDTA, EGTA and NTA. Logarithmic values of the pK value was plotted versus the pH value.

30

Figure 2: Agarose gel of HugI PCR amplification experiment. The lanes are annotated as follows: 1: DNA ladder , 2: MMA + 5 mM Mg, 3: MMA + 6 mM Mg, 4: MMA + 7 mM Mg, 5: MMA + 8 mM Mg, 6: MMA + 9 mM Mg, 7: MMA + 10 mM Mg, 8: DNA ladder, 9: DNA ladder, 10: MMB + 0.5 mM

Mg, 11: MMB + 1 mM Mg, 12: MMB + 2 mM Mg, 13: MMB + 3 mM Mg, 14: MMB + 4 mM Mg, 15: DNA ladder.

An increase in Mg concentration leads to successful amplification of the target product 'specific PCR product', although much unspecific product is visible (lane 10–lane 12). A further increase of Mg concentration leads to generation of unspecific PCR products (lane 14, 4 mM Mg Cl_2). In contrast, addition of 5 mM EGTA in presence of 5-10 mM $MgCl_2$ results in specific PCR product (lane 2 –lane 7), although the amount of unspecific by product increase while Mg concentration is increased.

Figure 3

Melting curves (EGTA titration experiment).

The curves are annotated as follows: A: no additive, B: 0.25 mM EGTA, C: 0.5 mM EGTA, D: 0.75 mM EGTA, E: 1 mM EGTA, F: 1.5 mM, G: 2 mM EGTA, H: 4-10 mM.

Figure 4

Melting curves (Mg titration experiment).

The curves are annotated as follows: A: no additives, B: 5 mM EGTA, C: 5 mM EGTA +0, 1 mM Mg, D: 5 mM EGTA + 0.2 mM Mg, E: 5 mM EGTA + 0.3 mM Mg, F: 5 mM EGTA + 0.4 mM Mg, G: 5 mM EGTA + 0.5 mM Mg, H: 5 mM EGTA + 0.6 mM Mg.

Figure 5

Agarose gel analysis of DNase assay at different temperatures and influence of EGTA.

Lanes are annotated as follows: Note that reactions corresponding to samples in lane 2-7 were performed on ice and are hence labelled 'on ice'. Similarly, reactions corresponding to samples in lanes 8-13 were performed at 42 °C and are labelled '42 °C' accordingly. Reactions at both temperatures were performed in the absence and presence of 100 µM EGTA ('0 µM EGTA and '100 µM EGTA respectively).

Lane M: GelPilot High Range Ladder (6 µl), lane 1: 1 µg WGA gDNA (no DNase added),

lanes 2 and 3: 1 µl DNase (1U) 5 and 15 min, lanes 4 and 5: 0.1 µl DNase (0.1U) 5 and 15 min, lanes 6 and 7: 0.01 µl DNase (0.01U), 5 and 15 min, lanes 8 and 9: 1 µl DNase (1U) 5 and 15 min, lanes 10 and 11: 0.1 µl DNase (0.1U) 5 and 15 min, lanes 12 and 13: 0.01 µl DNase (0.01U) 5 and 15 min.

Figure 6

Agarose gel analysis of DNase assay at different temperatures and influence of EDTA.
Reactions corresponding to samples in lanes 1-6 were performed on ice and are hence labeled 'on
5 ice' Reactions corresponding to samples in lane 7-12 were performed at 42 °C and are labeled '42 °C' accordingly. Lanes are annotated as follows: Lane K: 1 µg WGA gDNA.
Lane 1: 1 U DNase , lane 2: 0.5 U DNase , lane 3: 0.1 U DNase, Lane 4: 1 U DNase + 100 µM EDTA, lane 5: 0.5 U DNase + 100 µM EDTA, lane 6: 0.1 U DNase + 100 µM EDTA.
Lane 7: 1 U DNase, lane 8: 0.5 U DNase, lane 9: 0.1 U DNase, lane 10: 1 U DNase + 100 µM EDTA,
10 lane 11: 0.5 U DNase + 100 µM EDTA, lane 12: 0.1 U DNase + 100 µM EDTA, lane M: GelPilot 1 kb Ladder (3 µl).

Claims

1. Method for the regulation of enzyme activity in a reaction composition comprising the steps of:
   (i) providing a reaction composition comprising
      a. at least one enzyme, wherein the activity of said enzyme depends on the presence of divalent cations in the reaction composition,
      b. a divalent cation,
      c. a chelating agent wherein the binding of said cation to the chelating agent is dependent on pH and/or temperature of the reaction composition,
      d. a buffering system, wherein the acid dissociation constant is temperature dependent, such that a change in temperature results in a change of pH of the reaction composition,
      e. substrate for said enzyme and
   (ii) changing the temperature in the reaction composition, such that divalent cations which are bound to chelating agents are released from these complexes, wherein the enzyme is thereby activated or its activity is increased.

2. Method according to claim 1, wherein the enzyme is a nucleic acid modifying enzyme.

3. Method according to claims 1 to 3, wherein the nucleic acid modifying enzyme is selected from the group comprising polymerases, reverse transcriptases and nucleases.

4. Method according to claim 2, wherein the activity of said nucleic acid modifying enzyme comprises substrate binding and substrate processing.

5. Method according to claim 1 to 4, wherein the removal of said divalent cation from the nucleic acid modifying enzyme results in decreased activity or loss of activity.

6. Method according to any one of the preceding claims, wherein the chelating agent is selected from the group comprising ethylene diamine tetra acetate (EDTA), ethylene glycol bis(amino ethyl) N, N'-tetra acetate (EGTA) and nitrilo tri acetate (NTA).

7. Method according to any one of the preceding claims, wherein the divalent cation is selected from the group comprising Mg^{2+}, Ca^{2+}, Mn^{2+}, Cu^{2+}, Fe^{2+}, Ni^{2+}, Zn^{2+} and Co^{2+}.

8. Method according to any of the preceding claims, wherein the chelating agent is EDTA and the cations are selected from Mg^{2+}, Ca^{2+}, Mn^{2+}, Cu^{2+}, Ni^{2+}, Zn^{2+} and Co^{2+}.

9. Method according to any of the preceding claims, wherein the chelating agent is EGTA and the cations are Ca^{2+} and/or Mg^{2+}.

10. Method according to any of the preceding claims, wherein the chelating agent is NTA and the cations are $Ca^{2+}$and/or Cu^{2+} and/or Co^{2+}.

11. Method according to any one of the preceding claims, wherein the reaction composition comprises a buffer system, preferably a Tris buffer system; wherein the divalent cation is Mg^{2+}, preferably at a concentration between 0.01 and 20 mM; wherein the chelating agent is EGTA at a concentration between 0.05 and 50 mM and wherein the nucleic acid modifying enzyme is a DNA polymerase, preferably a hot start polymerase.

12. Method according to any one of the preceding claims, wherein the reaction composition comprises a buffer system, preferably a Tris buffer system; wherein the divalent cation is selected from the group of Mg^{2+}, Ca^{2+}, Mn^{2+}, Cu^{2+}, Fe^{2+}, Ni^{2+}, Zn^{2+} and Co^{2+}; wherein the chelating agent is selected from the group of EGTA, EDTA and NTA and wherein the nucleic acid enzyme is a nuclease.

13. Kit for performing a nucleic acid modifying reaction comprising:
    i. A buffer system
    ii. A chelating agent
    iii. A nucleic acid modifying enzyme
    iv. A divalent cation for said enzyme.

Figures

Figure 1

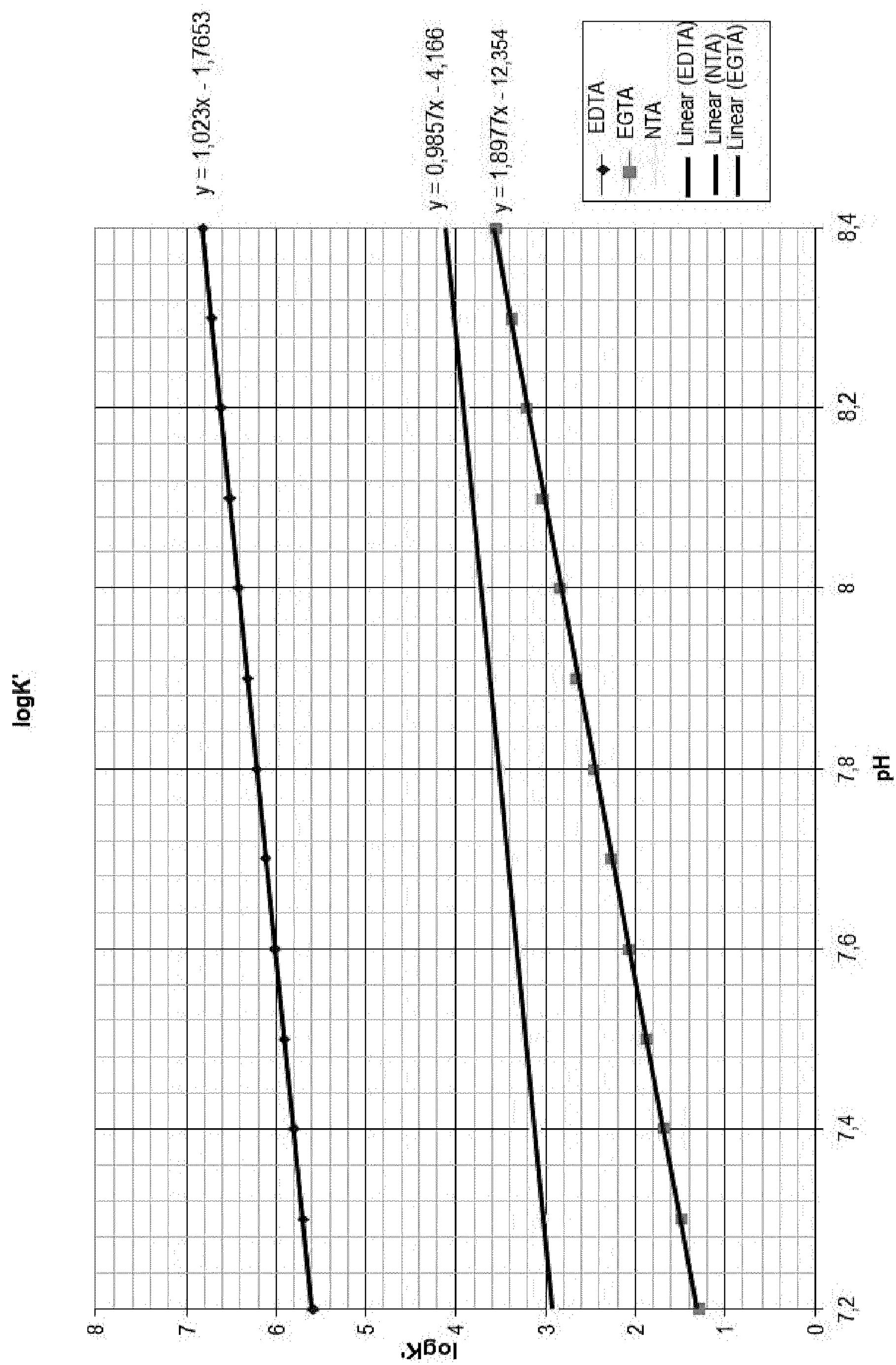


Figure 2

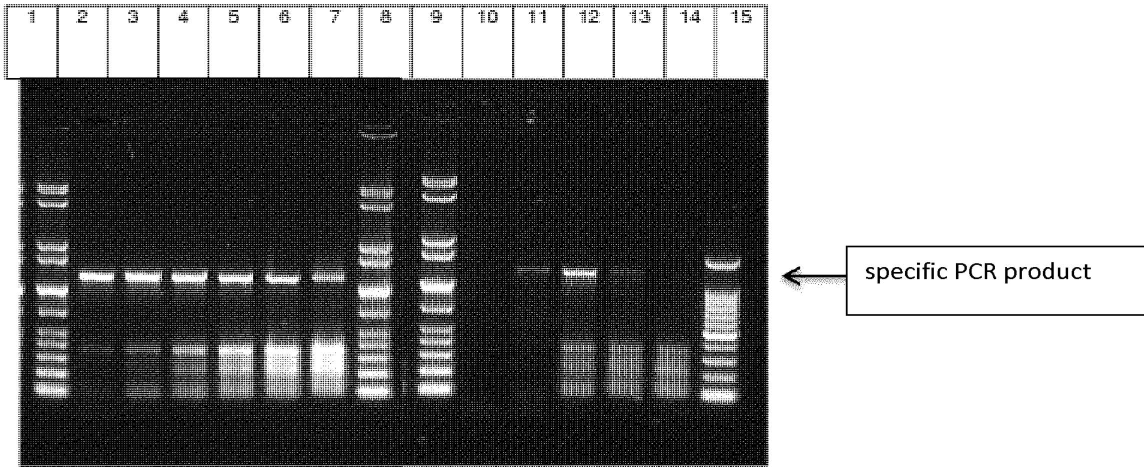
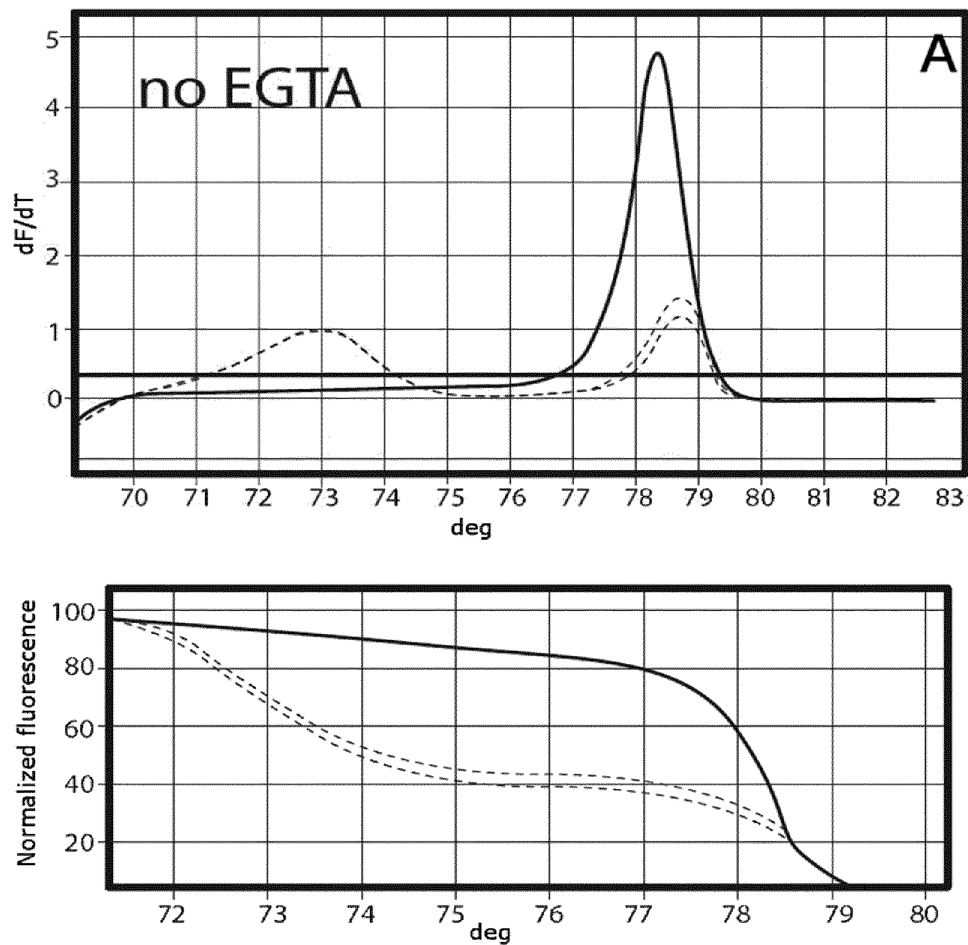


Figure 3



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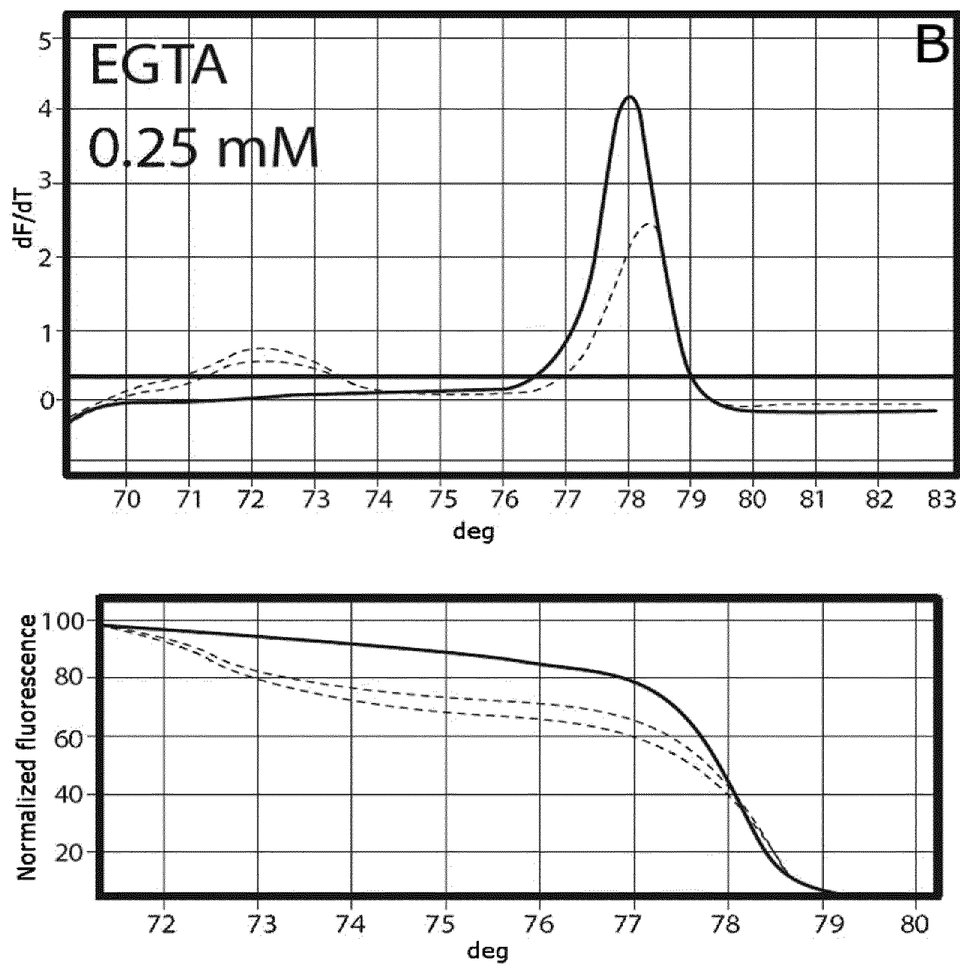

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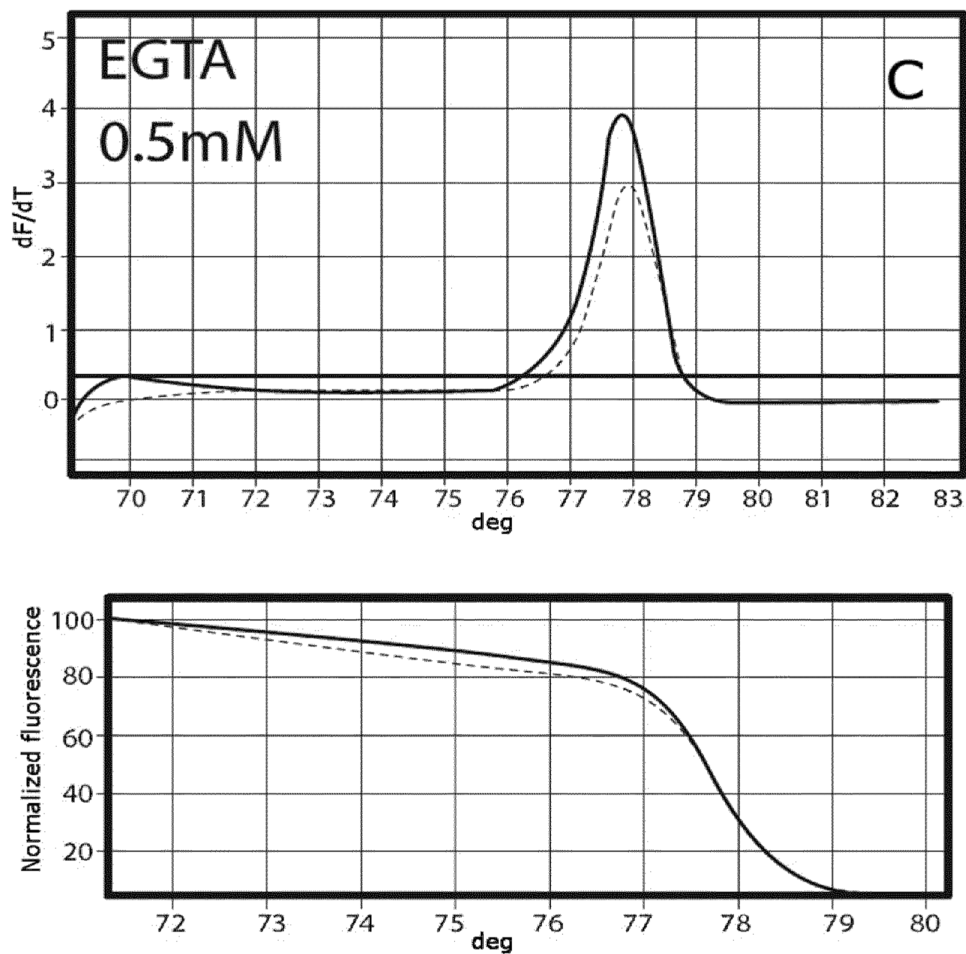

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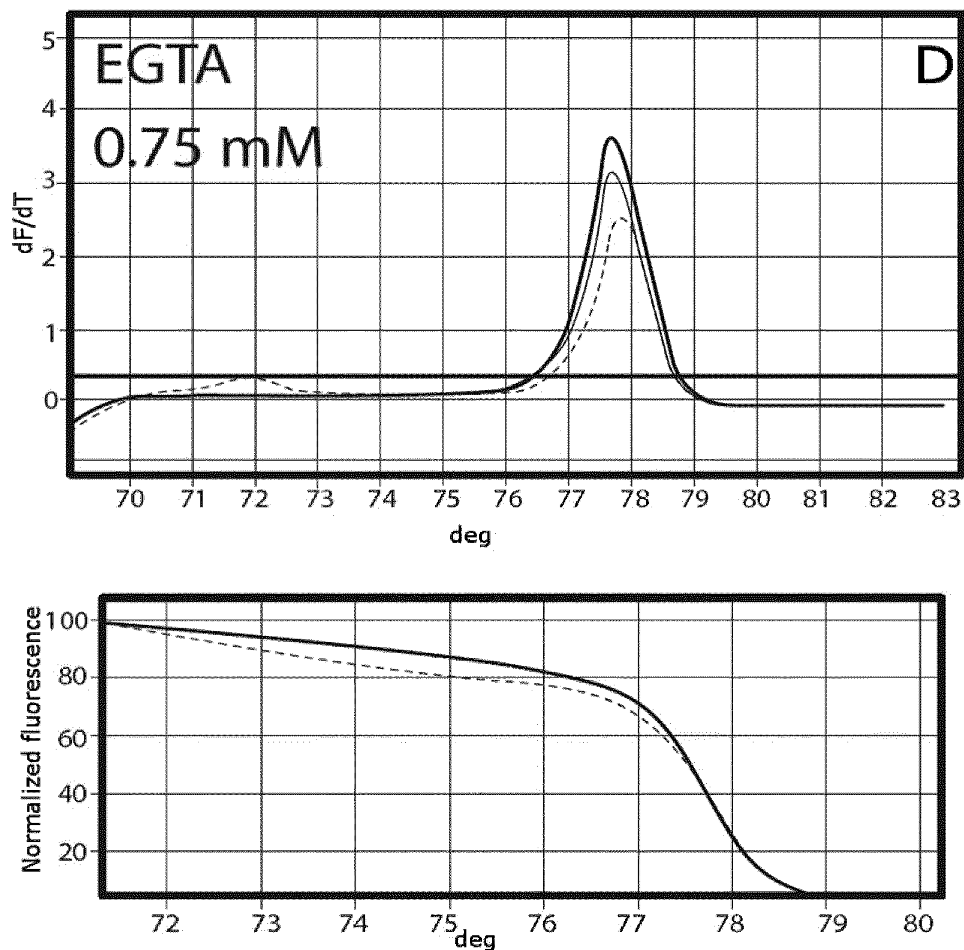

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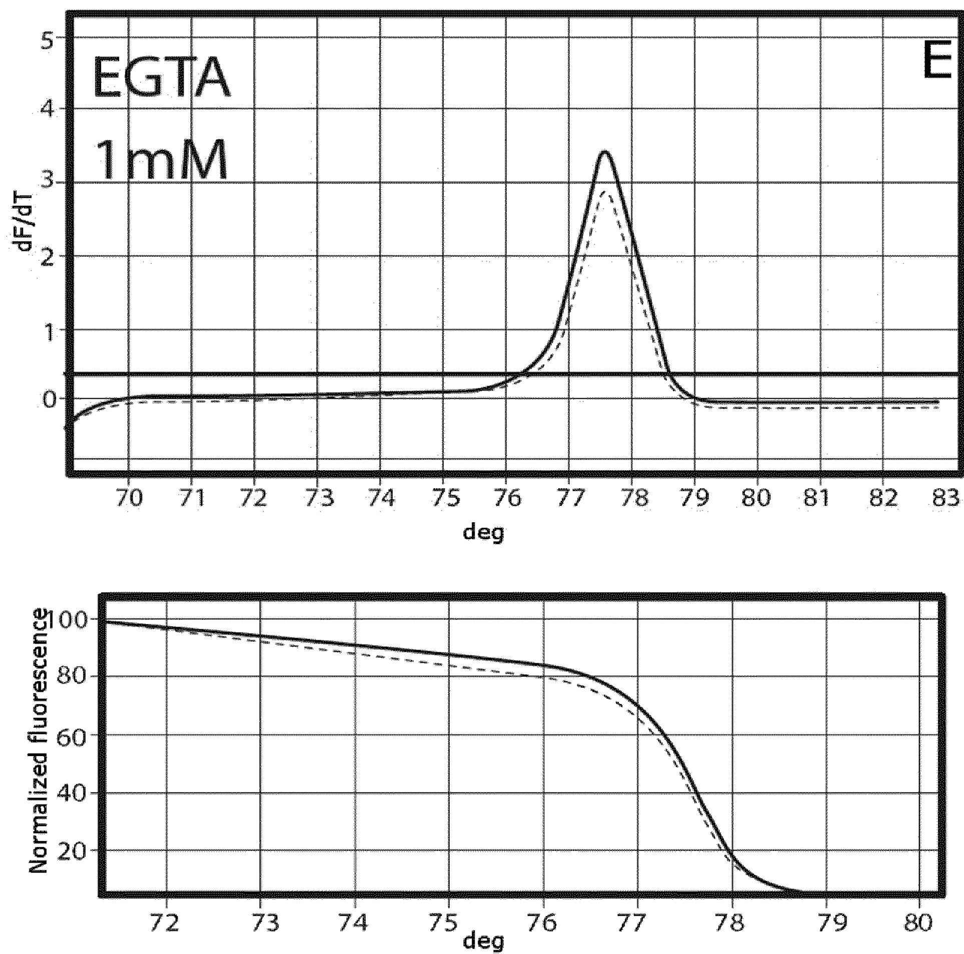

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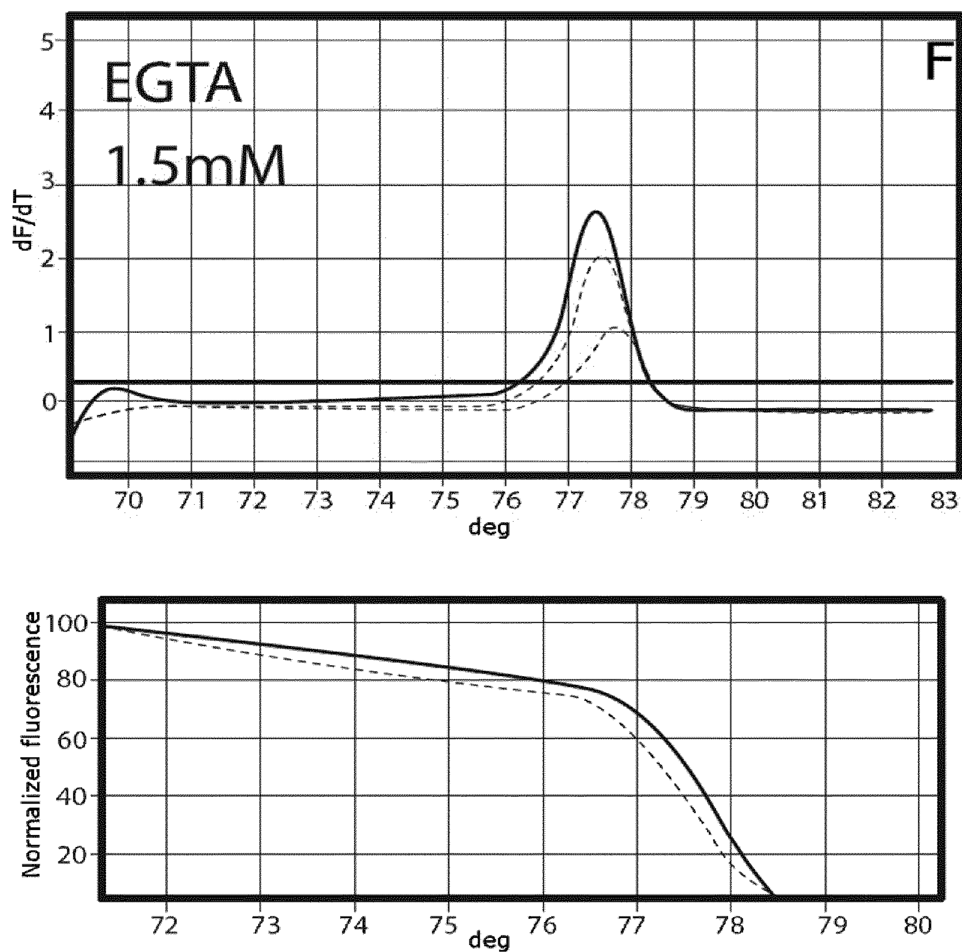

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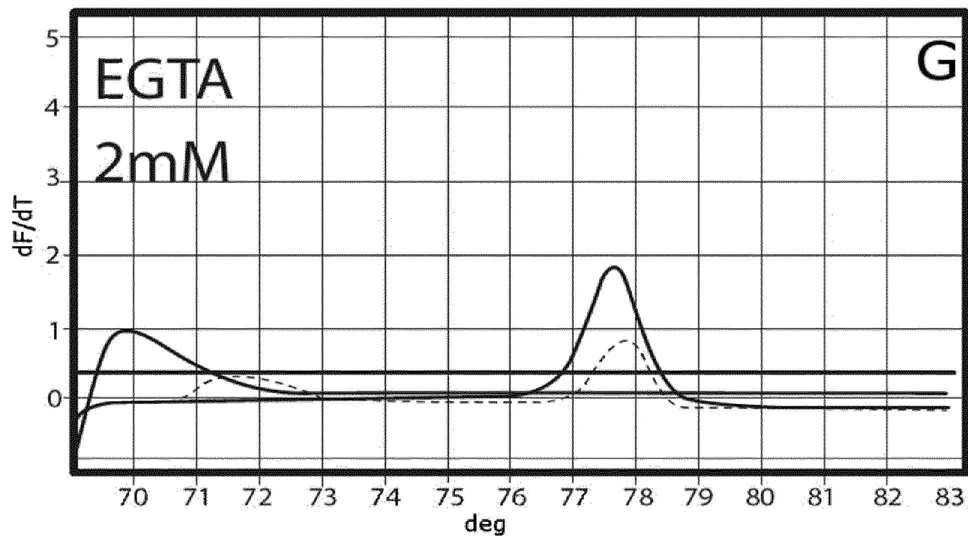

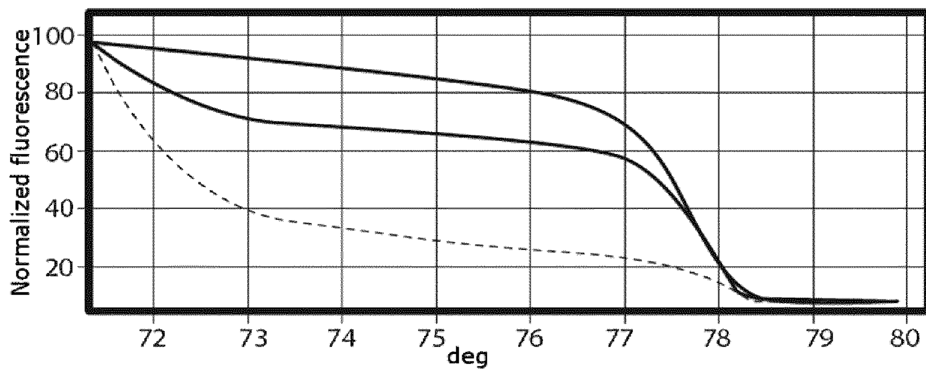

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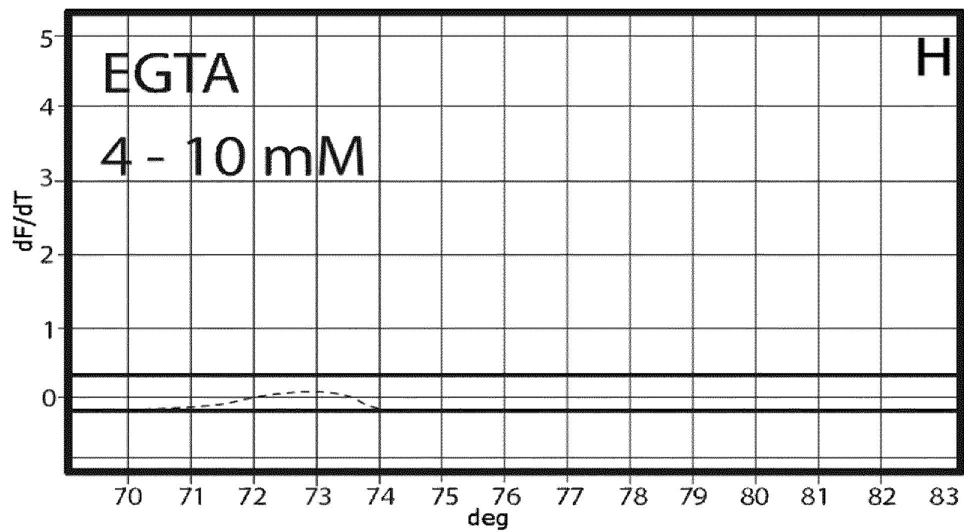


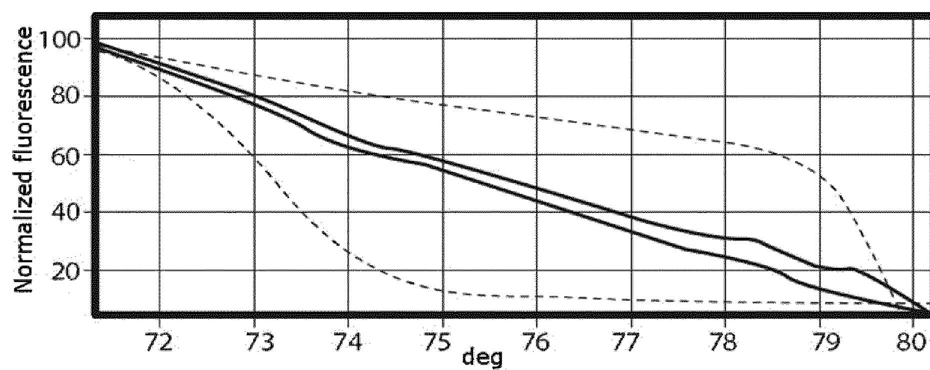
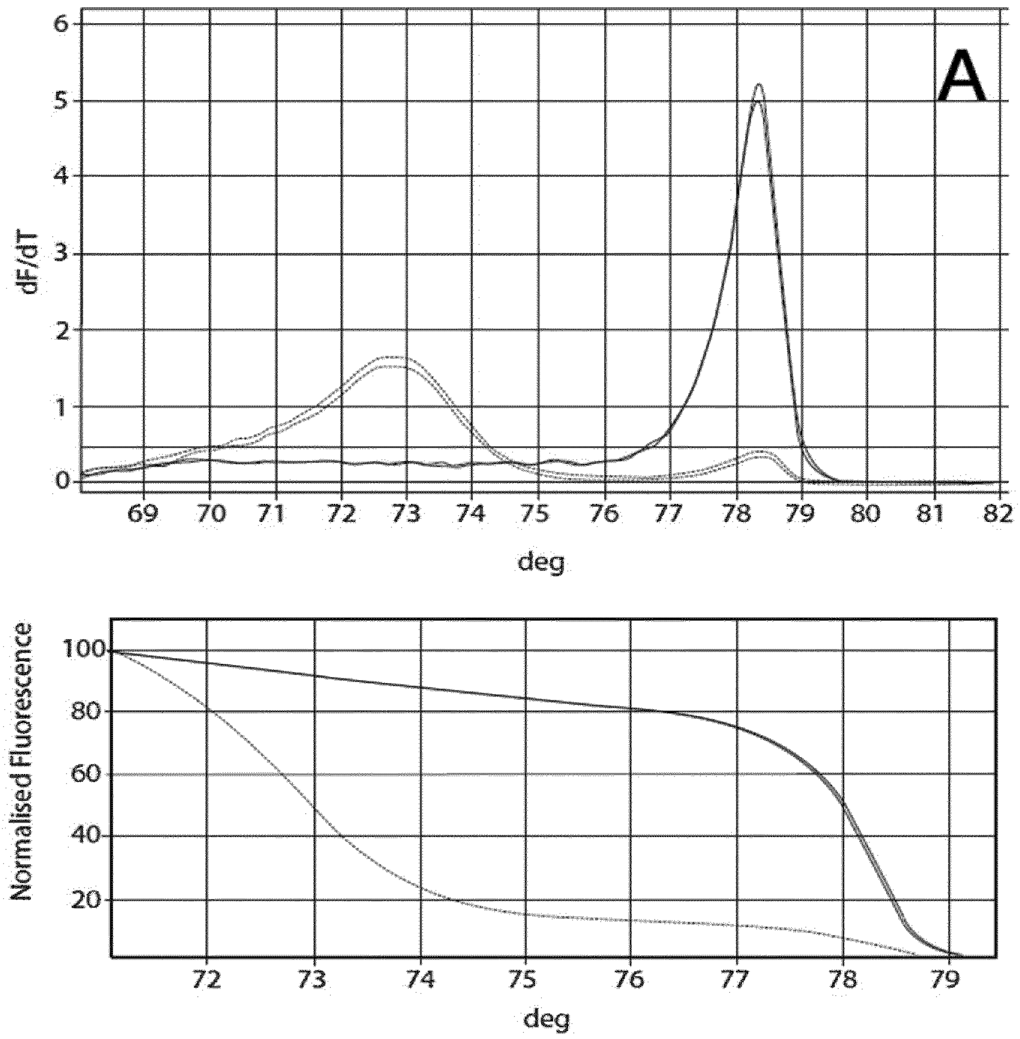


Figure 4



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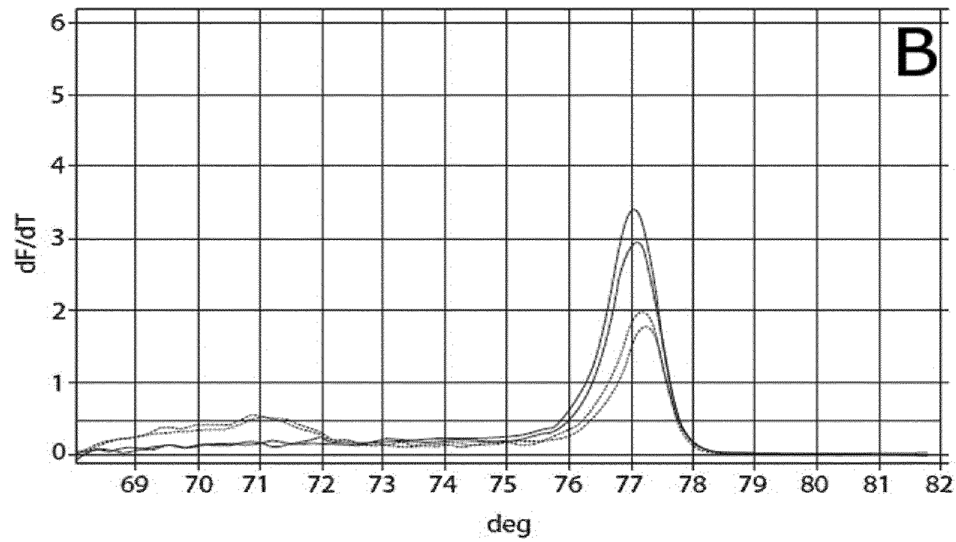


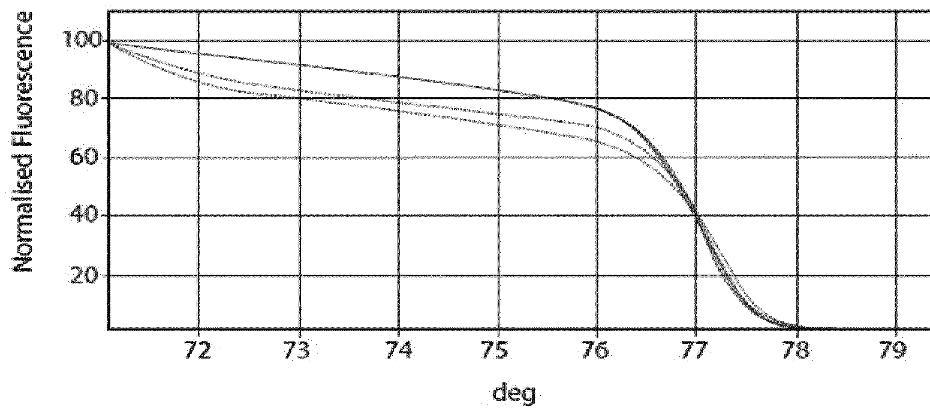
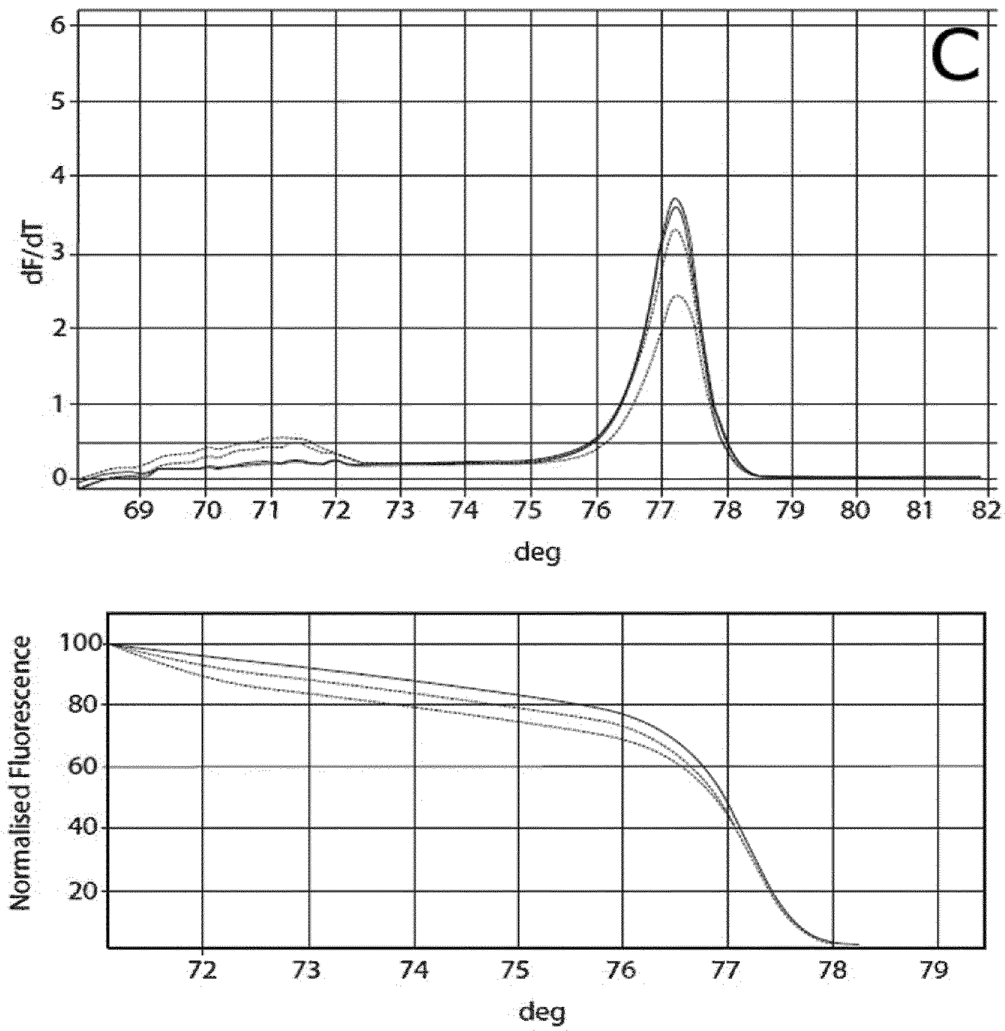


Figure 4 continued



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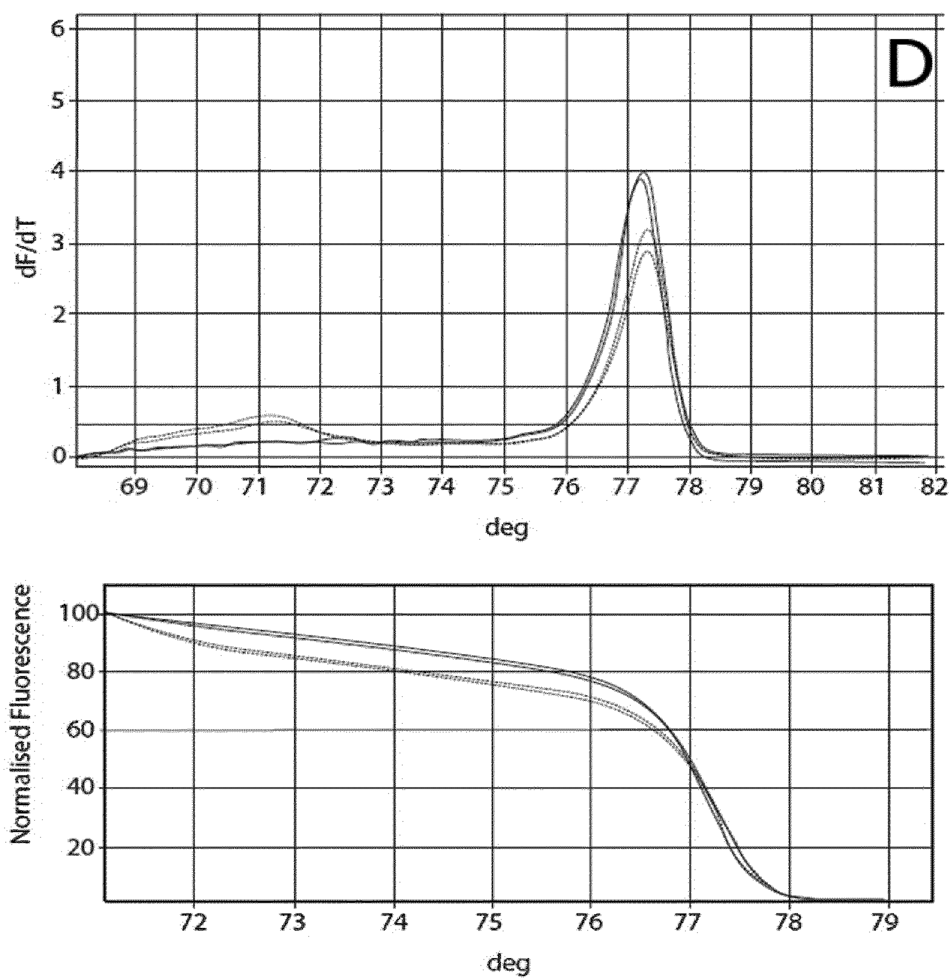

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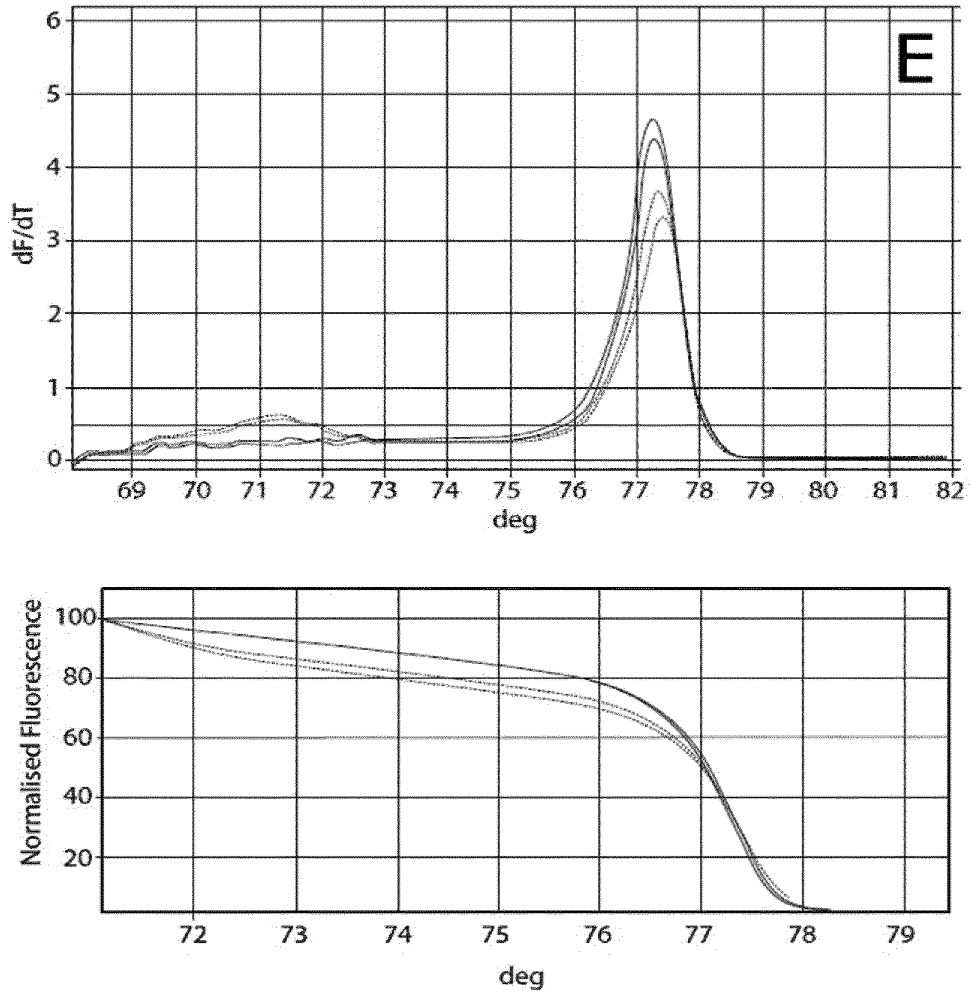

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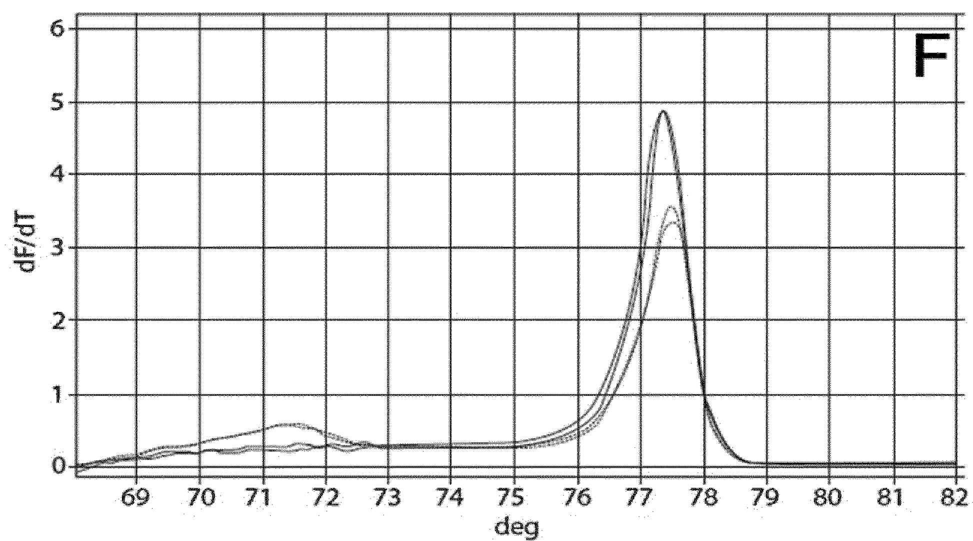

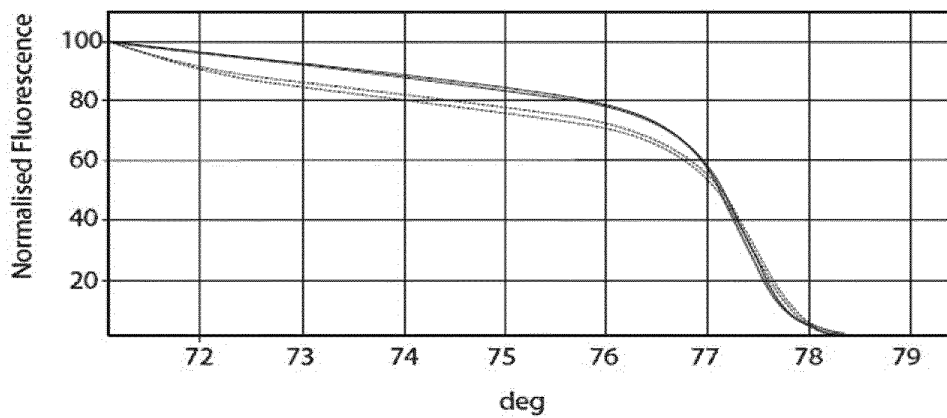

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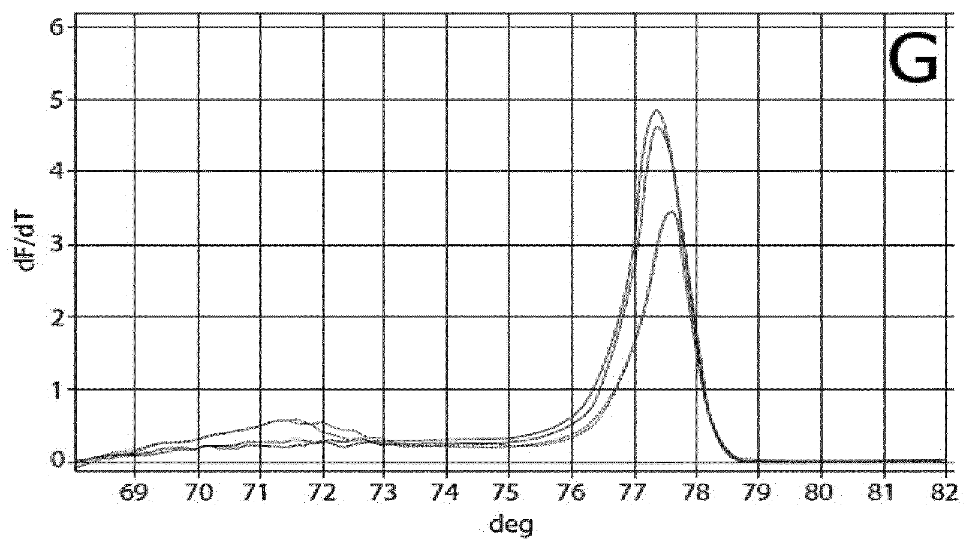

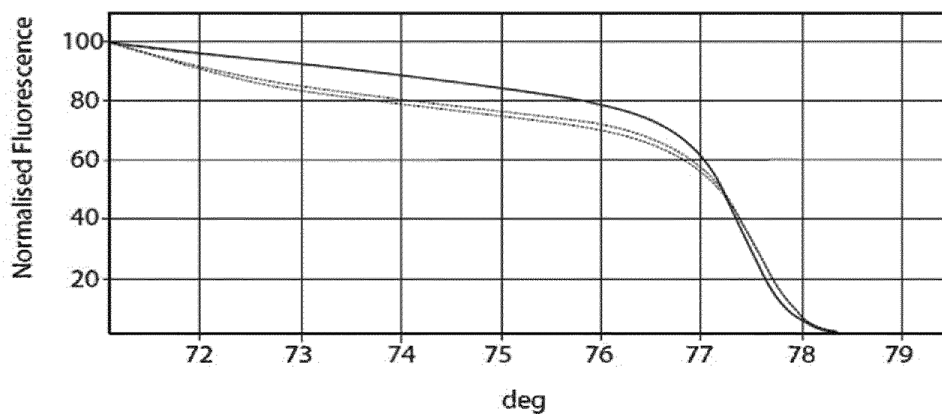

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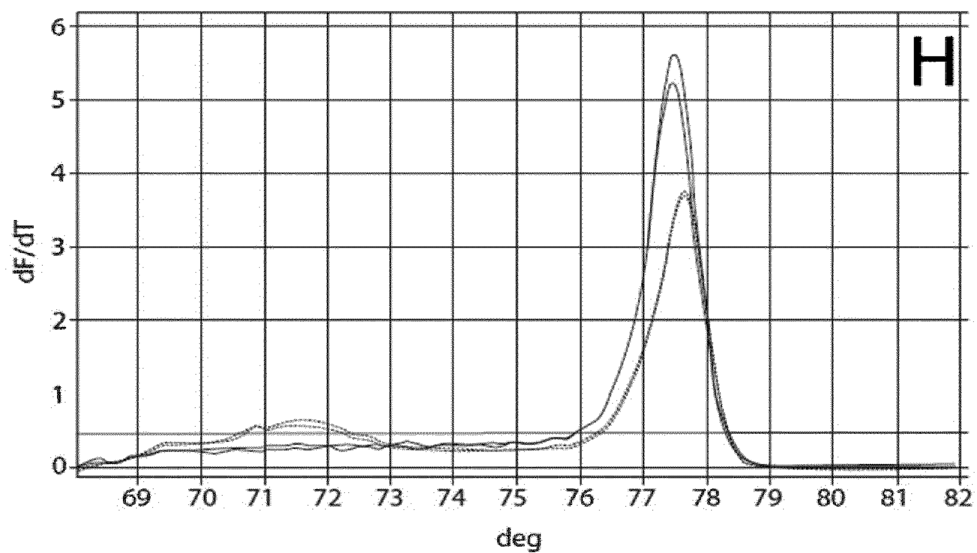


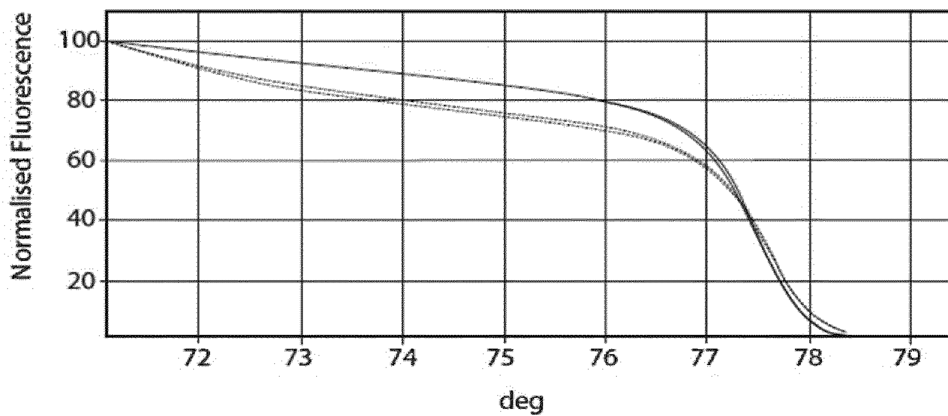


Figure 5

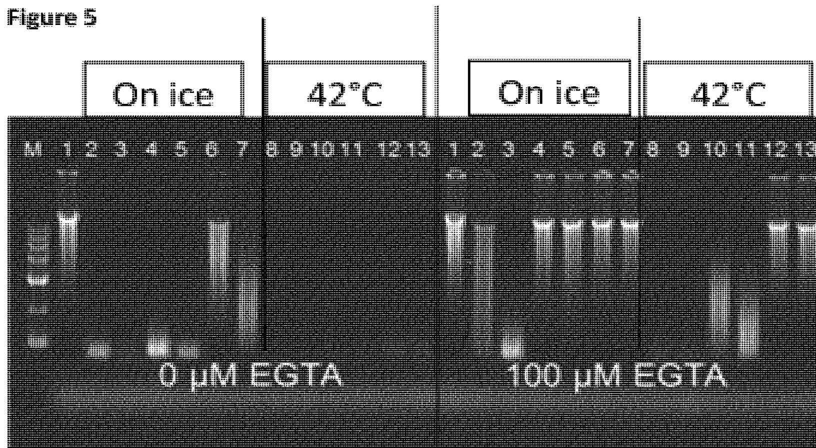
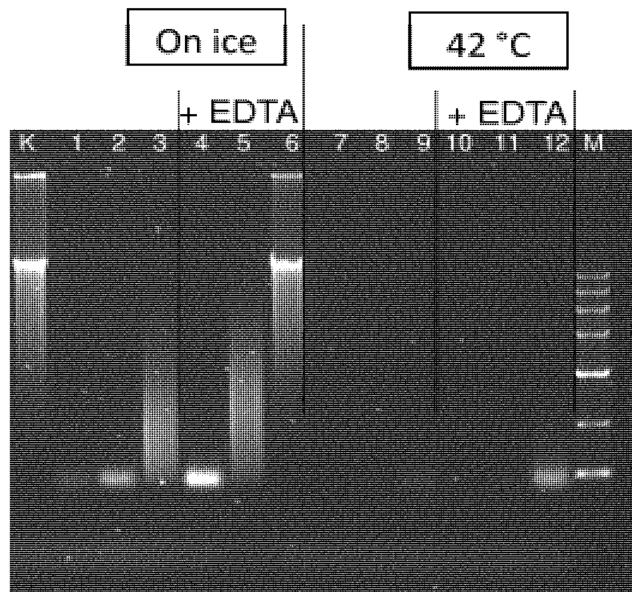


Figure 6



INTERNATIONAL SEARCH REPORT

International application No
PCT/EP2015/050671

A. CLASSIFICATION OF SUBJECT MATTER
INV. C12Q1/68
ADD.

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
C12Q

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

EPO-Internal, WPI Data, BIOSIS, CHEM ABS Data, Sequence Search, EMBASE

C. DOCUMENTS CONSIDERED TO BE RELEVANT

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Y	claim 1	6-12
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Y	page 5, lines 19-30; examples 1-4	6-12
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Y	paragraph [0067]	6-12

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[X] Further documents are listed in the continuation of Box C.

[X] See patent family annex.

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Date of the actual completion of the international search	Date of mailing of the international search report
26 February 2015	09/03/2015

Name and mailing address of the ISA/	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+31-70) 340-2040, Fax: (+31-70) 340-3016	Petri, Bernhard

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INTERNATIONAL SEARCH REPORT

International application No
PCT/EP2015/050671

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Y	figure 2; table II	6-12
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INTERNATIONAL SEARCH REPORT

Information on patent family members

International application No
PCT/EP2015/050671

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
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Form PCT/ISA/210 (patent family annex) (April 2005)

(12) INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(19) World Intellectual Property Organization
International Bureau

(10) International Publication Number
WO 2016/004333 A2

(43) International Publication Date
7 January 2016 (07.01.2016)

(51) International Patent Classification:
C12P 19/30 (2006.01) *C07H 21/04* (2006.01)

(21) International Application Number: PCT/US2015/039036

(22) International Filing Date: 2 July 2015 (02.07.2015)

(25) Filing Language: English

(26) Publication Language: English

(30) Priority Data:
62/020,227 2 July 2014 (02.07.2014) US

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(74) Agent: **STAPLE, David, W.**; 2275 Deming Way, Suite 310, Middleton, Wisconsin 53562 (US).

(81) Designated States *(unless otherwise indicated, for every kind of national protection available)*: AE, AG, AL, AM, AO, AT, AU, AZ, BA, BB, BG, BH, BN, BR, BW, BY, BZ, CA, CH, CL, CN, CO, CR, CU, CZ, DE, DK, DM, DO, DZ, EC, EE, EG, ES, FI, GB, GD, GE, GH, GM, GT, HN, HR, HU, ID, IL, IN, IR, IS, JP, KE, KG, KN, KP, KR, KZ, LA, LC, LK, LR, LS, LU, LY, MA, MD, ME, MG, MK, MN, MW, MX, MY, MZ, NA, NG, NI, NO, NZ, OM, PA, PE, PG, PH, PL, PT, QA, RO, RS, RU, RW, SA, SC, SD, SE, SG, SK, SL, SM, ST, SV, SY, TH, TJ, TM, TN, TR, TT, TZ, UA, UG, US, UZ, VC, VN, ZA, ZM, ZW.

(84) Designated States *(unless otherwise indicated, for every kind of regional protection available)*: ARIPO (BW, GH, GM, KE, LR, LS, MW, MZ, NA, RW, SD, SL, ST, SZ, TZ, UG, ZM, ZW), Eurasian (AM, AZ, BY, KG, KZ, RU, TJ, TM), European (AL, AT, BE, BG, CH, CY, CZ, DE, DK, EE, ES, FI, FR, GB, GR, HR, HU, IE, IS, IT, LT, LU, LV, MC, MK, MT, NL, NO, PL, PT, RO, RS, SE, SI, SK, SM, TR), OAPI (BF, BJ, CF, CG, CI, CM, GA, GN, GQ, GW, KM, ML, MR, NE, SN, TD, TG).

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WO 2016/004333 A2

(54) **Title**: REVERSIBLE METAL ION CHELATORS

(57) **Abstract**: Provided herein are chelator constructs (e.g., nucleic acid, peptide, peptide nucleic acid, etc.) that sequester metal ions (e.g., Mg^{2+}) under a first set of conditions and fail to sequester or release sequestered metal ions under a second set of conditions. In particular, nucleic acid constructs are provided that sequester metal ions (e.g., Mg^{2+}) under conditions that favor secondary and tertiary structure formation and release or fail to sequester metal ions under conditions that disfavor the formation of such structures.

REVERSIBLE METAL ION CHELATORS

CROSS-REFERENCE TO RELATED APPLICATIONS

The present invention claims priority to U.S. Provisional Patent Application Serial
5 Number 62/020,227, filed July 2, 2014, which is incorporated by reference in its entirety.

FIELD

Provided herein are chelator constructs that sequester metal ions under a first set of conditions and fail to sequester or release sequestered metal ions under a second set of
10 conditions.

BACKGROUND

Chelation involves the formation or presence of two or more separate coordinate bonds between a polydentate ligand and a single central metal ion. Chelators are used in a
15 variety of applications to remove metal ions from solution.

SUMMARY

Provided herein are chelator constructs (e.g., comprising nucleic acid, peptide, peptide nucleic acid, etc.) that sequester metal ions (e.g., Mg^{2+}, Zn^{2+}, Ca^{2+}, Mn^{2+}, etc.) under a first
20 set of conditions (e.g., low temperature, high salt, high pH, etc.) and fail to sequester or release sequestered metal ions under a second set of conditions (e.g., high temperature, low salt, neutral pH, etc.). In particular, biopolymers (e.g., nucleic acid constructs, peptide constructs, etc.) are provided that sequester metal ions (e.g., Mg^{2+}, Zn^{2+}, Ca^{2+}, Mn^{2+}, etc.) under conditions that favor secondary and tertiary structure formation and release or fail to
25 sequester metal ions under conditions that disfavor the formation of such structures.

In some embodiments, provided herein are reversible chelator constructs comprising one or more structural moieties attached to two or more chelation components (e.g., 2, 3, 4, 5, 6, 7, 8, etc.), wherein under a first set of conditions (e.g., high temperature conditions) said one or more structural moieties adopt a first conformation that positions and/or orients said
30 two or more chelation components such that said two or more chelation components are unable to efficiently chelate metal ions (e.g., chelate less efficiently), and wherein under a second set of conditions (e.g., low temperature conditions) said one or more structural moieties adopt a second conformation that positions and/or orients said two or more chelation components such that said two or more chelation components efficiently chelate metal ions

(e.g., more efficiently chelate metal ions). In some embodiments, the one or more structural moieties are selected from peptides, nucleic acids, and peptide-nucleic acids. In some embodiments, the one or more structural moieties are nucleic acids.

In some embodiments, the reversible chelator construct comprises a single structure-forming nucleic acid strand and two chelation components, wherein formation of the structure brings the chelation components into appropriate proximity and orientation to efficiently chelate metal ions and wherein melting of the structure separates the chelation components such that said chelation components are unable to efficiently chelate metal ions. In some embodiments, the chelation components are attached to 5' and 3' termini of the nucleic acid strand. In some embodiments, the chelation components are attached to the nucleic acid strand internally. In some embodiments, chelation components are attached to the nucleic acid strand by a linker. In some embodiments, the structure is selected from a stem-loop, hairpin, cruciform, triple helix, pseudoknot, two-stem junctions, etc.

In some embodiments, the reversible chelator construct comprises a first biopolymer (e.g., first nucleic acid strand) attached to a first chelation component and a second biopolymer (e.g., second nucleic acid strand attached to a second chelation component), wherein the first and second nucleic acid strands are complementary, wherein hybridization of the nucleic acid strands brings the chelation components into appropriate proximity and orientation to efficiently chelate metal ions, and wherein melting of the nucleic acid strands separates the chelation components such that said chelation components are unable to efficiently chelate metal ions. In some embodiments, the first chelation component is attached to the 5' end of the first nucleic acid strand and the second chelation component is attached to the 3' end of the second nucleic acid strand. In some embodiments, the chelation components are attached to the nucleic acid strands internally. In some embodiments, chelation components are attached to the nucleic acid strands by linkers.

In some embodiments, the reversible chelator construct comprises a nucleic acid strand that is not attached to a chelation component and one or more nucleic acid strands attached chelation components, wherein hybridization of the one or more nucleic acid strands to chelation components to the nucleic acid strand not attached to a chelation component brings the chelation components into appropriate proximity and orientation to efficiently chelate metal ions, wherein melting of the nucleic acid strands separates the chelation components such that said chelation components are unable to efficiently chelate metal ions.

In some embodiments, the two or more chelation components are iminodiacetic acid moieties, A23187 moieties, phosphate (e.g., a phosphate moiety added to a structural moiety,

a 5' phosphate of a nucleic acid strand, etc.), acetic acid, polyether (PEG), thioether, thiol, amine, heterocyclic amine such as pyridine, bipyridine, terpyridine, phenanthroline, etc. In some embodiments, a chelation moiety comprises any compound, functional group, molecule, macromolecule, ionophore, etc. that is (1) capable of efficiently chelating one or more types of metal ions, and (2) divisible into two or more chelation components that are separately incapable of efficient metal-ion chelation, but are capable of efficient metal-ion chelation when properly positioned (e.g., within a particular distance) and oriented.

In some embodiments, high temperature is a temperature above a transition temperature between the first and second conformations. In some embodiments, low temperature is a temperature below a transition temperature between the first and second conformations.

In some embodiments, less efficient chelation is a K_d of 10 μM or more, 50 μM or more, 100 μM or more, 200 μM or more, 500 μM or more, or 1 mM or more. In some embodiments, more efficient chelation is a K_d of 1 μM or less, 500 nM or less, 200 nM or less, 100 nM or less, 50 nM or less, 10 nM or less, or 1 nm or less.

In some embodiments, less efficient chelation refers to an affinity for metal ions that is less (e.g., 2-fold less, 3-fold less, 4-fold less, 5-fold less, 10-fold less, 20-fold less, 50-fold less, 100-fold less, 1000-fold less, etc.) than the affinity of the other components of the system or reaction (e.g., DNA polymerase, DNA, nucleotides, etc.) for the sample metal ions. In some embodiments, more efficient chelation refers to an affinity for metal ions that is greater (e.g., 2-fold more, 3-fold more, 4-fold more, 5-fold more, 10-fold more, 20-fold more, 50-fold more, 100-fold more, 1000-fold more, etc.) than the affinity of the other components of the system or reaction (e.g., DNA polymerase, DNA, nucleotides, etc.) for the sample metal ions.

In some embodiments, the affinity of a chelator construct for a metal ion (e.g., Mg^{2+}, Zn^{2+}, Ca^{2+}, Mn^{2+}, etc.) in the chelation conformation (e.g., more efficient chelation conformation) is at least one log higher affinity (e.g., >2 logs higher affinity, >3 logs higher affinity, >4 logs higher affinity, >5 logs higher affinity, >6 logs higher affinity, >7 logs higher affinity, >8 logs higher affinity, or more) than in the release or non-chelation conformation (e.g., more efficient chelation conformation). In some embodiments, the affinity of a chelator construct for a metal ion (e.g., Mg^{2+}, Zn^{2+}, Ca^{2+}, Mn^{2+}, etc.) in the chelation conformation (e.g., more efficient chelation conformation) is between two and six logs higher than in the release or non-chelation conformation (e.g., more efficient chelation conformation).

In some embodiments, the provided herein are methods of regulating an enzymatic reaction comprising: (a) providing in a reaction mix: (i) an enzyme that catalyzes said enzymatic reaction, wherein said enzyme employs a metal ion cofactor for catalysis; (ii) a substrate for said enzyme; (iii) optionally any other reactants for said enzymatic reaction; (iv) the metal ion cofactor for said enzyme; and (v) a reversible chelator construct that is incapable of efficiently chelating said metal ion cofactor under a first set of conditions and more efficiently chelates said metal ion cofactor under a second set of conditions; (b) exposing said reaction mix to said first set of conditions, wherein said enzyme less efficiently catalyzes said enzymatic reaction (e.g., reaction rate is $<1/2V_{max}$, $<1/4V_{max}$, $<1/8V_{max}$, $<1/16V_{max}$, $<1/32V_{max}$, $<1/64V_{max}$, $<1/100V_{max}$, $<1/1000V_{max}$, or less) under said first set of conditions because said metal ion cofactor is chelated by the reversible chelator construct; and (c) exposing said reaction mix to said second set of conditions, wherein said enzyme catalyzes said enzymatic reaction under said second set of conditions because said metal ion cofactor is available in solution.

In some embodiments, step (b) is performed before step (c). In some embodiments, the method further comprises: (d) repeating step (b).

In some embodiments, step (c) is performed before step (b). In some embodiments, the method further comprises: (d) repeating step (c).

In some embodiments, the enzyme is DNA polymerase said metal ion cofactor is magnesium. In some embodiments, the enzymatic reaction is polymerase chain reaction. In some embodiments, the enzymatic reaction is cleavage of nucleic acids (e.g., 3' to 5').

In some embodiments, inefficient catalysis (or less efficient catalysis) is less than 50% enzyme activity (e.g., of maximum enzyme activity), less than 40% enzyme activity, less than 30% enzyme activity, less than 20% enzyme activity, less than 10% enzyme activity, less than 50% enzyme activity, or less than 1% enzyme activity. In some embodiments, efficient catalysis (or more efficient catalysis) is greater than 50% enzyme activity (e.g., of maximum enzyme activity), greater than 60% enzyme activity, greater than 70% enzyme activity, greater than 80% enzyme activity, greater than 90% enzyme activity, greater than 95% enzyme activity, greater than 99% enzyme activity.

In some embodiments, inefficient catalysis is defined as the absence of detectable reaction products (e.g., PCR products, secondary PCR products, 5' to 3' nuclease product, 3'to 5' nuclease product, restriction digest product, etc.). In some embodiments, inefficient catalysis is defined as reduction of detectable reaction products (e.g., PCR products, secondary PCR products, 5' to 3' nuclease product, 3'to 5' nuclease product, restriction

digest product, etc.) compared to the reaction performed in the absence of a chelator construct (e.g., <50% of product, <10% of product, <1% of product, <0.5% of product, <0.1 product, etc.) . In some embodiments, the amount of detectable reaction products is determined by standard detection methods for the particular reaction. In some embodiments, efficient catalysis is defined as a comparable amount of detectable reaction products (e.g., PCR products, secondary PCR products, 5' to 3' nuclease product, 3'to 5' nuclease product, restriction digest product, etc.) compared to the reaction performed in the absence of a chelator construct (e.g., >50%, >75%, >90%, >95%, >99%, >100%, or more). In some embodiments, the amount of detectable reaction products is determined by standard detection methods for the particular reaction (e.g., gel electrophoresis).

In some embodiments, provided herein are compositions comprising one or more biopolymers and two or more iminodiacetic acid moieties, wherein upon folding and/or hybridization of said one or more biopolymers said two or more iminodiacetic acid moieties are positioned adjacent to one another. In some embodiments, the one or more biopolymers are selected from nucleic acids, peptides, and peptide nucleic acids. In some embodiments, the two or more iminodiacetic acid moieties are attached to the same biopolymer of said one or more biopolymers. In some embodiments, the two or more iminodiacetic acid moieties are attached to the separate biopolymers of said one or more biopolymers.

BRIEF DESCRIPTION OF THE DRAWINGS

FIG. 1 shows illustrations of exemplary nucleic acid chelator constructs under hybridization/folding conditions, in which chelation components are brought together to form chelator moieties: (A) two nucleic acid structural moieties, both end-labeled with chelator components; (B) three nucleic acid structural moieties, two of which end-modified with chelator components; (C) two nucleic acid structural moieties, both internally-modified with chelator components; and (D) a single nucleic acid structural moiety, internally-modified at two positions with chelator components. In each case, when these exemplary constructs are placed in conditions that disfavor hybridization/folding, the chelation components cease to be in close proximity. (E) shows an illustration of base pairing in a double stranded DNA and the dimensions thereof.

FIGS. 2A-I show illustrations of exemplary chelators comprising nucleic acid structural moieties. For each construct (A-I), the conditions on the left favor secondary structure formation and the conditions on the right disfavor secondary structure. (A) Two complementary nucleic acid strands, one with a 5' chelation component and the other with a

3' chelation component that form a chelation moiety when the stands are hybridized. (B) Two complementary nucleic acid strands, each with 5' and 3' chelation components that form two chelation moieties when the stands are hybridized. (C) Two complementary nucleic acid strands, both with internal chelation components that form a chelation moiety when the stands are hybridized. (D) One hairpin forming nucleic acid strand, with 5' and 3' chelation components that form a chelation moiety when the hairpin is formed. (E) One hairpin-forming nucleic acid strand, with two internal chelation components that form a chelation moiety within the stem when the hairpin is formed. (F) One hairpin-forming nucleic acid strand, with internal chelation components that form a chelation moiety within the loop when the hairpin is formed. (G) One pseudoknot-forming nucleic acid strand, with internal chelation components that form a chelation moiety when the pseudoknot is formed. (H) One double-hairpin-forming nucleic acid strand, with 5' and 3' chelation components that form a chelation moiety at a two-stem junction under folding conditions. (I) A first nucleic acid strand with a 5' chelation component, a second nucleic acid strand with a 3' chelation component, and third nucleic acid strand without chelation components; the first and second nucleic acid strands hybridize to the third, forming a chelation moiety when the stands are hybridized.

FIG. 3 shows exemplary structural moieties.

FIG. 4 shows an exemplary application of the chelator constructs described herein; a plurality of chelators are used to create a metal ion gradient across a surface.

FIG. 5 shows an exemplary chelator construct comprising three nucleic acid structural moieties.

FIG. 6 shows an exemplary chelator construct comprising two nucleic acid structural moieties.

FIGS. 7A-D show the application of the reversible metal ion chelators to polymerase and 3' to 5' nuclease degradation in PCR. A) With reversible metal ion chelators and at low temperature, the chelators sequesters magnesium rendering the DNA polymerase inactive. B) With reversible metal ion chelators and at high temperature, the magnesium is released and able to activate the DNA polymerase and PCR proceeds. C) Without the reversible metal ion chelators and at low temperature, the magnesium is able to activate the DNA polymerase. At this temperature the polymerase domain of the DNA polymerase can start polymerization of secondary products and primer dimers and the 3' to 5' nuclease domain can degrade primers and template DNA. D) Without the reversible metal ion chelators and at high

temperature, the amplification quality is affected by accumulation of secondary products, primer dimers and decreased yield.

DEFINITIONS

5 As used herein, the term “chelation moiety” refers to a compound or functional group containing electron-donating groups that can combine by coordinative bonding with a metal ion to form a stable structure. Typically, a chelation moiety is part of a larger molecule or macromolecule. In some embodiments, a chelation moiety comprises two separate compounds or functional groups (e.g., chelation components).

10 As used herein, the term “chelation component” refers to a compound or functional group, that when brought in close proximity to and/or proper orientation with one or more other chelation components, forms a chelation moiety. A single chelation component is not capable of efficient or stable chelation, as it has insufficient affinity for the metal ion to sequester it (e.g., from participating in a chemical or enzymatic reaction), whereas the full 15 chelation moiety has sufficient affinity. In some embodiments, a chelation moiety is part of a larger molecule or macromolecule.

As used herein, the term “chelator” or “chelator construct” refers to a compound, polymer, complex, or other molecular or macromolecular entity containing electron-donating groups that can combine by coordinative bonding with a metal ion to form a stable structure. 20 A chelator may consist solely of a moiety capable of chelation (e.g., chelation moiety) or may further comprise other molecular or macromolecular portions (e.g., “structural moieties”). In some embodiments, a chelator comprises two or more compounds, polymers, complexes, or other molecular or macromolecular entities that when combined create a structure capable of chelating a metal ion.

25 As used herein, the term “structural moiety” refers to a compound, polymer, or other molecular or macromolecular entity that adopts secondary, tertiary, and/or quaternary structure under appropriate conditions.

The term “nucleic acid”, as used herein, refers to an oligonucleotide, nucleotide or polynucleotide, and fragments or portions thereof, and to DNA or RNA of genomic or 30 synthetic origin which may be single or double stranded, and represent the sense or antisense strand.

The term “nucleic acid strand” refers to a single continuous polymer of nucleotides. A single-stranded nucleic acid is one nucleic acid stand. A double stranded nucleic acid is two complementary nucleic acid stands.

The term "stem loop", also called "hairpin loop", refers to a structure that comprises a double-stranded portion (stem), formed by hydrogen bonding between inverted repeat sequences in a single-stranded nucleic acid molecule, and a loop portion sandwiched in between. A stem may be formed of 2 to 100 base pairs, and a loop may be formed of 3 to 20 nucleotides.

The terms “peptide” and “polypeptide” as used herein refer to polymers of amino acids, of any length and of natural or synthetic origin.

The term “amino acid” refers to naturally occurring and non-naturally occurring amino acids, as well as imino acids such as proline, amino acid analogs and amino acid mimetics that function in a manner similar to the naturally occurring amino acids. Amino acids comprise a central carbon, connected to H, amine, carboxylic acid, and R groups. Typical amino acids differ only at the R position, and the identity of the amino acid (e.g., glycine, alanine, tyrosine, etc.) is defined by the R position. Other modifications of amino acids are within the scope of the invention.

Naturally encoded amino acids are the proteinogenic amino acids known to those of skill in the art. They include the 20 common amino acids (alanine, arginine, asparagine, aspartic acid, cysteine, glutamine, glutamic acid, glycine, histidine, isoleucine, leucine, lysine, methionine, phenylalanine, proline, serine, threonine, tryptophan, tyrosine, and valine) and the less common pyrrolysine and selenocysteine. Naturally encoded amino acids include post-translational variants of the 22 naturally occurring amino acids such as prenylated amino acids, isoprenylated amino acids, myrisoylated amino acids, palmitoylated amino acids, N-linked glycosylated amino acids, O-linked glycosylated amino acids, phosphorylated amino acids and acylated amino acids. The term "non-natural amino acid" refers to an amino acid that is not a proteinogenic amino acid, or a post-translationally modified variant thereof. In particular, the term refers to an amino acid that is not one of the 20 common amino acids or pyrrolysine or selenocysteine, or post-translationally modified variants thereof.

“Solid support” refers herein to any substrate having a surface to which molecules may be attached, directly or indirectly, through either covalent or non-covalent bonds. A “solid support” can have a variety of physical formats, which can include, for example, a membrane; a chip (e.g., a protein chip); a slide (e.g., a glass slide or coverslip); a column; a hollow, solid, semi-solid, pore- or cavity- containing particle, such as, for example, a bead; a gel; a fiber, including a fiber optic material; a matrix; and a sample receptacle. Exemplary sample receptacles include sample wells, tubes, capillaries, vials, and any other vessel, groove or indentation capable of holding a sample. A sample receptacle can be contained on

a multi-sample platform, such as a microtiter plate, slide, microfluidics device, and the like. A support can be composed of a natural or synthetic material, an organic or inorganic material. The composition of the solid support on which capture reagents are attached generally depends on the method of attachment (e.g., covalent attachment). Other exemplary
5 receptacles include microdroplets and microfluidic controlled or bulk oil/aqueous emulsions within which assays and related manipulations can occur. Suitable solid supports include, for example, plastics, resins, polysaccharides, silica or silica-based materials, functionalized glass, modified silicon, carbon, metals, inorganic glasses, membranes, nylon, natural fibers (such as, for example, silk, wool and cotton), polymers, and the like. The material
10 composing the solid support can include reactive groups such as, for example, carboxy, amino, or hydroxyl groups, which are used for attachment of the capture reagents. Polymeric solid supports can include, e.g., polystyrene, polyethylene glycol tetraphthalate, polyvinyl acetate, polyvinyl chloride, polyvinyl pyrrolidone, polyacrylonitrile, polymethyl methacrylate, polytetrafluoroethylene, butyl rubber, styrenebutadiene rubber, natural rubber,
15 polyethylene, polypropylene, (poly)tetrafluoroethylene, (poly)vinylidenefluoride, polycarbonate, and polymethylpentene.

DETAILED DESCRIPTION

Provided herein are chelator constructs (e.g., comprising nucleic acid, peptide, peptide
20 nucleic acid, etc.) that sequester metal ions (e.g., Mg^{2+}, Zn^{2+}, Ca^{2+}, etc.) under a first set of conditions (e.g., low temperature, high salt, high pH, etc.) and fail to sequester or release sequestered metal ions under a second set of conditions (e.g., high temperature, low salt, neutral pH, etc.).

In certain embodiments, provided herein are constructs (e.g., single polymer, duplex,
25 complex, etc.) that form a metal-ion-chelating structure (e.g., low K_d (e.g. nanomolar, micormolar, etc.)) under a first set of conditions (e.g., structure forming conditions (e.g., low temperature, high salt, neutral pH)) and denature or form a structure that does not chelate metal ions (e.g., high K_d (e.g., micromolar, millimolar, etc.)) under a second set of conditions (e.g., conditions that do not favor the formation of secondary and/or tertiary structures (e.g.,
30 high temperature, low salt, high pH)).

Under a first set of conditions (e.g., one or more of high temperature, low salt, and/or high pH, etc.) the chelator construct is unfolded and the chelation components dissociate. For example, under the first set of conditions, a structural moiety or moieties of a chelator construct are denatured and unable to form a tertiary structure necessary/sufficient to properly

align/orient the chelation components for efficient chelation. Likewise, under the first set of conditions, a structural moiety or moieties (e.g., nucleic acid strand or strands) of a chelator construct are unhybrized and unable to form a secondary structure necessary/sufficient to properly align/orient the chelation components for efficient chelation. In some embodiments, the first set of conditions includes a temperature above the melting temperature (T_m) of the secondary, tertiary, or quaternary structure of the structural moiety or moieties (e.g., $>T_m+1$°C, $>T_m+2$°C, $>T_m+3$°C, $>T_m+4$°C, $>T_m+5$°C, $>T_m+6$°C, $>T_m+7$°C, $>T_m+8$°C, $>T_m+9$°C, $>T_m+10$°C, $>T_m+15$°C, $>T_m+20$°C, or more).

Under a second set of conditions (e.g., one or more of low temperature, high salt, and/or neutral pH, etc.), the chelator construct is folded and the chelation moiety is formed. For example, under the second set of conditions, a structural moiety or moieties of a chelator construct form a tertiary structure necessary/sufficient to properly align/orient the chelation components for efficient chelation. Likewise, under the second set of conditions, a structural moiety or moieties (e.g., nucleic acid strand or strands) of a chelator construct are hybrized and able to form a secondary structure necessary/sufficient to properly align/orient the chelation components for efficient chelation. In some embodiments, the second set of conditions include a temperature below the melting temperature (T_m) of the secondary, tertiary, or quaternary structure of the structural moiety or moieties (e.g., $<T_m-1$°C, $<T_m-2$°C, $<T_m-3$°C, $<T_m-4$°C, $<T_m-5$°C, $<T_m-6$°C, $<T_m-7$°C, $<T_m-8$°C, $<T_m-9$°C, $<T_m-10$°C, $<T_m-15$°C, $<T_m-20$°C, or less).

In some embodiments, a chelator construct comprises one or more structural moieties and one or more chelation moieties. Any combination of structural and chelation moieties (e.g., 1:2, 2:1, 2:2, 2:4, 3:2, etc.) are within the scope of the invention.

In some embodiments, a chelator construct comprises two structural moieties, each connected to a chelation component (e.g., a functional group that does not efficiently chelate metal ions alone, but does efficiently chelate metal ions when combined with a second chelation component to form a chelation moiety). When the chelator construct is exposed to conditions that promote folding and/or hybridization, the chelation components are brought together allowing the chelation of metal ions (e.g., allowing efficient chelation of metal ions, low K_d). When the chelator construct is exposed to conditions that disfavor folding and/or hybridization, the chelation components are pulled apart (on their respective structural moieties) or reoriented, preventing the chelation components from chelating metal ions (e.g., preventing efficient chelation of metal ions, high K_d).

In some embodiments, a chelator construct comprises two chelation components connected to different portions of a single structural moiety (e.g., opposite ends of the structural moiety). When the chelator construct is exposed to conditions that promote folding and/or hybridization, the chelation components are brought together by the folding of the structural moiety, thereby forming a chelation moiety and allowing the chelation of metal ions (e.g., allowing efficient chelation of metal ions, low K_d). When the chelator construct is exposed to conditions that disfavor folding and/or hybridization, the chelation moiety is pulled apart into the individual chelation components (on their respective portions of the structural moiety), preventing the chelation of metal ions (e.g., preventing efficient chelation of metal ions, high K_d).

In some embodiments, a chelation moiety forms (e.g., from two or more chelation components)), to provide a single site for chelation of metal ions on a chelator construct. In other embodiments, a single chelator construct (comprising one or more structural moieties) comprises multiple sites for metal ion chelation (e.g., multiple chelator moieties or pairs of chelation components).

B. Chelation moieties

In certain embodiments, a chelation moiety comprises two or more separate chelation components (e.g., iminodiacetic acid moieties) that form a chelation moiety under a first set of conditions, but do not form a chelation moiety under a second set of conditions. In some embodiments, the chelation components are separated in physical space or by orientation under non-chelation conditions but are brought together or re-oriented under chelation conditions to form a chelation moiety. In such embodiments, the ability of the chelation moiety to chelate metal ions is modulated by forming and/or denaturing the chelation moiety. Suitable chelation moieties that find use in such embodiments are compounds, functional groups, moieties, etc. that chelate magnesium ions, including, but not limited to: iminodiacetic acid groups (See, e.g., Trapani et al. *Analyst*, 2010, 135, 1855–1866; herein incorporated by reference in its entirety). Other chelation moieties comprise A23187 moieties, phosphate (e.g., a phosphate moiety added to a structural moiety, a 5' phosphate of a nucleic acid strand, etc.), acetic acid, polyether (PEG), thioether, thiol, amine, heterocyclic amine such as pyridine, bipyridine, terpyridine, phenanthroline, etc.

In some embodiments, two or more chelation components are oriented under chelation conditions to form or approximate a known metal-ion chelator. Suitable chelation moieties that are formed or approximated in such embodiments include, but are not limited

to: diethylenetriaminepentaacetic acid (DTPA), dimercaprol, ethylenediaminetetraacetic acid (EDTA), EDTA analogs (US 2002/0182227; herein incorporated by reference in its entirety), tetraazacyclododecanetetraacetic acid (DOTA), 2,3-Dimercapto-1-propanesulfonic acid (DMPS), dimercaptosuccinic acid (DMSA), α-Hydroxytropolones (WO 2007065007;
5 herein incorporated by reference in its entirety), penicillamine, deferoxamine, deferasirox, and other chelator moieties that incorporate electron donating atoms such as O, S, P or N as Lewis bases to bind the metal (Engelstad and Wolf, "Contrast Agents", in Magnetic Resonance Imaging, Stark and Bradley, Mosby, St. Louis, 1988, pp. 161-181; herein incorporated by reference in its entirety). In some embodiments, chelation moieties are
10 obtained from other metal binding constructs (See, e.g., Carter et al. Chem. Rev. 2014, 114, 4564−4601; Que et al. Chem Rev. 2008 May;108(5):1517-49.; Hyman and Franz. Coordination Chemistry Reviews 256 (2012) 2333– 2356.; herein incorporated by reference in their entireties).

In some embodiments, a chelation moiety comprises multiple (e.g., two)
15 iminodiacetic acid components which are brought together (e.g., through folding of a structural moiety and/or hybridization of structural moieties) to form an efficient cation sequesterer (e.g., simulating the structure of EDTA), but do not chelate cations (e.g., efficiently) when apart. In some embodiments, the folding of a structural moiety and/or hybridization of structural moieties brings the iminodiacetic acid components into close
20 enough proximity and/or into the appropriate orientation to efficiently chelate a metal ion (e.g., Mg^{2+}).

In some embodiments, a chelation moiety or chelation components for a construct are selected to chelate specific metal ions. For example, two iminodiacetic acid components brought together sequester Ca^{2+}, Fe^{3+}, and/or Mg^{2+}; chelator components brought together to
25 form a DOTA-like structure sequester Gd^{3+}; chelator components brought together to form a dimercaprol-like structure sequester arsenic, gold, lead, and/or copper; etc. Depending upon the intended application of the chelator construct, different chelation moieties and/or chelation components are selected.

In some embodiments, a chelator moiety chelates many types of metal ion. In other
30 embodiments, more specific chelator moieties are used. In some embodiments, chelator moieties are used that chelate one or more of: lithium, sodium, potassium, rubidium, cesium, francium, beryllium, magnesium, calcium, strontium, barium, radium, aluminum, gallium, indium, tin, thallium, lead, bismuth, manganese, iron, cobalt, nickel, copper, zinc, yttrium,

zirconium, niobium, molybdenum, technetium, ruthenium, rhodium, palladium, silver, cadmium, lanthanum, hafnium, tantalum, tungsten, rhenium, osmium, iridium, platinum, gold, or mercury. In some embodiments, a chelator moiety is provided that chelates one or more of magnesium, iron, calcium, manganese, etc. In some embodiments, a magnesium ion
5 chelator is provided.

In some embodiments, chelation moieties and/or components are attached to structural moieties directly (e.g., by covalent linkage). In other embodiments, chelation moieties and/or components are attached to structural moieties indirectly or by a linker. Embodiments are not limited to any particular linker moiety. In some embodiments, the
10 linker connects two moieties (e.g. chelation components and structural moiety). In some embodiments, a linker moiety is cleavable (e.g., chemically cleavable, enzyme cleavable, etc.), such that exposure to appropriate conditions (e.g., cleaving enzyme) cleaves the linker moiety and separates the connected moieties. In some embodiments, the linker moiety is a covalent linkage that is: linear, branched, cyclic, heterocyclic, saturated, unsaturated, or
15 various combinations thereof. In some embodiments, the linker comprises 1-100 non-hydrogen atoms (in addition to hydrogen atoms) selected from the group of C, N, P, O and S (e.g. 1-75, 1-50, 1-40, 1-30, 1-20, 1-10, 1-5, etc.). In some embodiments, the linker comprises any combination of alkyl, ether, thioether, polyether, amine, alkyl, amide, ester, carboxamide, sulfonamide, hydrazide bonds and aromatic or heteroaromatic bonds. In some embodiments,
20 the linker comprises a polymer (e.g. nucleic acid, polypeptide, lipid, or polysaccharide), a peptide linker, a modified peptide linker, a Poly(ethylene glycol) (PEG) linker, a streptavidin-biotin or avidin-biotin linker, polyaminoacids (e.g., polylysine), functionalized PEG, polysaccharides, glycosaminoglycans, dendritic polymers such as described in WO93/06868 and by Tomalia et al. in Angew. Chem. Int. Ed. Engl. 29:138-175 (1990), PEG-chelant
25 polymers such as described in W94/08629, WO94/09056 and WO96/26754, oligonucleotide linker, phospholipid derivatives, alkenyl chains, alkynyl chains, disulfide, or a suitable combination thereof. In some embodiments, a linker moiety comprises any covalent or noncovalent molecular connector capable of stably stringing together a first and second moiety. One of ordinary skill in the art will further appreciate that the above linkers are not
30 intended to be limiting.

C. Structural moieties

In some embodiments, chelators provided herein comprise structural moieties attached to chelation components that form chelation moieties and sequester metal ions under certain conditions. In some embodiments, structural moieties undergo structural alterations

that result in modulation of chelation by regulating formation/deformation of the chelation moiety (from chelation components).

1. Nucleic acid

In some embodiments, a chelator comprises one or more nucleic acid structural moieties (See, e.g., FIG. 1). In some embodiments, a single chelation component is attached to a nucleic acid strand (See, e.g., FIG. 1A-C). In some embodiments, two or more (e.g., 2, 3, 4, 5, 6, 7, 8, 9, 10, or more) chelation components are attached to a nucleic acid strand (See, e.g., FIG. 1D). In some embodiments, a nucleic acid strand without an attached chelation moiety or component(s) is a structural moiety (e.g., interacting with another structural moiety or structural moieties that are attached to chelation components; See, e.g., FIG. 1D).

Chelation components are attached (e.g., directly or indirectly (e.g., via a linker)) to nucleic acid structural moieties at any suitable position or location that allows for modulation of chelation under varying conditions. In some embodiments, chelation components are attached to the backbone of the nucleic acid strand (e.g., phosphate group, sugar (e.g., ribose, deoxyribose, etc.), etc.). In some embodiments, chelation moieties/components are attached to a base of the nucleic acid strand (e.g., at a modified base). In some embodiments, chelation moieties/components are attached at the terminus of a nucleic acid strand (e.g., 3' OH, 5' phosphate, base, etc.). In some embodiments, chelation moieties/components are attached at an internal position of a nucleic acid strand (e.g., sugar (e.g., ribose, deoxyribose, etc.), base, etc.).

In some embodiments, one or more modified nucleotides incorporated into a nucleic acid structural moiety allow for attachment of chelation components. Exemplary modified nucleotides include those with 5' acrylic phosphoramidite; 5' adenylation; 3'-, 5'-, or internal NHS ester; etc. Other suitable modified nucleotides that are used, in some embodiments, to attach chelation moieties and/or components to structural moieties include, but are not limited to: 4-acetylcytosine, 8-hydroxy-N6-methyladenosine, aziridinylcytosine, pseudoisocytosine, 5-(carboxyhydroxylmethyl) uracil, 5-fluorouracil, 5-bromouracil, 5-carboxymethylaminomethyl-2-thiouracil, 5-carboxymethylaminomethyluracil, dihydrouracil, inosine, N6-isopentenyladenine, 1-methyladenine, 1-methylpseudouracil, 1-methylguanine, 1-methylinosine, 2,2-dimethylguanine, 2-methyladenine, 2-methylguanine, 3-methylcytosine, 5-methylcytosine, N6-methyladenine, 7-methylguanine, 5-methylaminomethyluracil, 5-methoxyaminomethyl-2-thiouracil, beta-D-mannosylqueosine, 5'-methoxycarbonylmethyluracil, 5-methoxyuracil, 2-methylthio-N6-isopentenyladenine, uracil-5-oxyacetic acid methylester, uracil-5-oxyacetic acid, oxybutoxosine, pseudouracil, queosine,

2-thiocytosine, 5-methyl-2-thiouracil, 2-thiouracil, 4-thiouracil, 5-methyluracil, N-uracil-5-oxyacetic acid methylester, uracil-5-oxyacetic acid, pseudouracil, queosine, 2-thiocytosine, and 2,6-diaminopurine.

In some embodiments, chelation components are attached to two separate nucleic acid structural moieties (See, e.g., FIG. 2A-C). Under hybridization conditions (e.g., low temperature, high salt concentration, neutral pH), the structural moieties hybridize, bringing the chelation components into close proximity and the appropriate orientation to form a chelation moiety and allow metal-ion chelation. In such embodiments, chelation of metal ions (e.g., efficient chelation of metal ions) is dependent upon the two chelation components being in close proximity and/or adopting a particular orientation. Chelator components in such embodiments are attached at locations on the separate strands that render them unable to chelate metal ions (e.g., incapable of efficient chelation) when the strands are not hybridized, but capable of chelation (e.g., efficient chelation) when the strands are hybridized. In some embodiments, chelation components are located at the 5' and 3' ends of complementary strands, such that they are adjacent when the strands hybridize (See, e.g., FIG. 2A-B). In such embodiments, strands may comprise chelation components at both 3' and 5' ends, such that the duplex has chelation moieties at both ends (See, e.g., FIG. 2B), or each stand comprises a single chelation component to produce a duplex with one chelation moiety (See, e.g., FIG. 2A). In other embodiments, chelation components are within the nucleic acid strands such that the duplex comprises a chelation moiety internal to the strand (See, e.g., FIG. 2C).

In some embodiments, chelation components are attached to two separate locations on a single nucleic acid structural moiety (See, e.g., FIG. 2D-H). Under hybridization and/or folding conditions (e.g., low temperature, high salt concentration, neutral pH), the nucleic acid structural moiety adopts a secondary, tertiary, and/or quaternary structure that brings the chelation components into close proximity and/or into an orientation that allows for formation of a chelation moiety. The present invention is not limited by the types of structures that allow for modulation of chelation. For example, in certain embodiments, under hybridization/folding conditions a nucleic acid structural moiety forms a stem-loop (See, e.g., FIG. 2D-F), pseudoknot (See, e.g., FIG. 2G), triplex, hairpin, bulge loop (e.g., unpaired bases on one side of a helix), interior loop (e.g., unpaired bases on both sides of a helix), two-stem junction (FIG. 2H), etc. Further, the present invention is not limited by the mechanisms through which the secondary, tertiary, or quaternary structure modulates chelation. In some embodiments, chelation components on the 5' and 3' ends of a nucleic

acid structural moiety from a chelation moiety when the nucleic acid strand forms a stem-loop (e.g., at low temperature), but separate when the stem-loop is melted (e.g., at high temperature) (See, e.g., FIG. 2D). In other embodiments, chelation components located internally with a nucleic acid strand form a chelation moiety within the stem of a stem-loop, 5 but dissociate when the stem-loop melts (See, e.g., FIG. 2E). In other embodiments, chelation components located internally with a nucleic acid strand form a chelation moiety within the loop of a stem-loop, but dissociate when the stem-loop melts (See, e.g., FIG. 2F). In some embodiments, chelation components located internally with a nucleic acid strand form a chelation moiety upon formation of a pseudoknot, but dissociate when the stem-loop 10 melts (See, e.g., FIG. 2G). In some embodiments, chelation components on the 5' and 3' ends of a nucleic acid structural moiety from a chelation moiety when the nucleic acid strand forms two stem-loops (e.g., at low temperature) thereby placing the chelation components at a two-stem junction, but separate when the stem-loop is melted (e.g., at high temperature) (See, e.g., FIG. 2H).

15 In some embodiments, a chelator construct comprises a structural moiety without any attached chelation components or chelation moieties, that interacts (e.g., hybridizes) with other structural moieties to modulate chelation (See, e.g., FIG. 2I). For example, in some embodiments, 3' and 5' chelator-components labeled oligonucleotides hybridize to a structural moiety without any attached chelation components to form a two-stem junction that 20 places the chelator components within proximity and in the proper orientation to form a chelator moiety (See, e.g., FIG. 2I). In some embodiments, the chelation moiety on one of the structural moieties (e.g., the 5' chelator-component labeled oligonucleotide) is a phosphate group intrinsic to the oligonucleotide. In some embodiments, the chelation moiety is a 5' phosphate of a nucleic acid.

25 ***2. Peptide***

In some embodiments, a structural moiety is a peptide or polypeptide. In some embodiments, a structural moiety is a synthetic peptide or polypeptide. In some embodiments, two or more chelation components attached to a single peptide or polypeptide structural moiety are brought together in proper orientation upon folding of the peptide or 30 polypeptide. In other embodiments, chelation components attached to separate peptides and/or polypeptides are brought together to form a chelation moiety upon interaction of the peptides and/or polypeptides.

Chelation components are attached to peptide/polypeptide structural moieties at any suitable position or location that allows for modulation of chelation under varying conditions.

In some embodiments, chelation components are attached to an amino acid (e.g., at a modified base). In some embodiments, a synthetic amino acid comprises a chelation component as the R group. In some embodiments, chelation constructs are formed by incorporation of chelation component containing amino acids into peptides and/or
5 polypeptides. In some embodiments, chelation components are attached at the C- and/or N-terminus of a peptide or polypeptide. In some embodiments, chelation moieties/components are attached at an internal position of a peptide or polypeptide strand.

In some embodiments, chelation components are attached to modified or unnatural amino acids incorporated into a peptide or polypeptide. Suitable modified or unnatural amino
10 acids include, but are not limited to: alanine derivatives, alicyclic amino acids, arginine derivatives, aromatic amino acids, asparagine derivatives, aspartic acid derivatives, beta-amino acids, cysteine derivatives, DAB (2,4-diaminobutyric acid), DAP (2,3-diaminopropionic acid), glutamic acid derivatives, glutamine derivatives, glycine derivatives, histidine derivatives, homo-amino acids, isoleucine derivatives, leucine derivatives, linear
15 core amino acids, lysine derivatives, methionine derivatives, n-methyl amino acids, norleucine derivatives
norvaline derivatives, ornithine derivatives, penicillamine derivatives, phenylalanine derivatives, phenylglycine derivatives, proline derivatives, pyroglutamine derivatives, serine derivatives, threonine derivatives, tryptophan derivatives, tyrosine derivatives, valine
20 derivatives, etc. (Biochemicals & Reagents for Life Science Research (2004-2005); herein incorporated by reference in their entireties).

In some embodiments, formation of a chelation moiety is modulated by folding/unfolding of the secondary, tertiary, and/or quaternary structure of one or more peptide/polypeptide structural moieties. Any suitable structures and/ or interactions of
25 structures may be utilized to modulate chelation within the scope of the invention.

In some embodiments, the formation of protein secondary structure regulates formation of a chelation moiety and modulates chelation. For example, in some embodiments, the formation of an alpha helix, 3_{10} helix, or π helix brings chelation components together in the proper orientation to form a chelation moiety, but unfolding of
30 the helix separates or misorients the chelation components. In some embodiments, the formation of a beta strand or beta sheet brings chelation components together in the proper orientation to form a chelation moiety, but unfolding of the structure separates or misorients the chelation components. In such embodiments, switching conditions from those that favor

secondary structure formation to those that disfavor it (as well as the reverse) allows modulation of chelation efficiency.

In some embodiments, the formation of protein tertiary structure regulates formation of a chelation moiety and modulates chelation. The present invention is not limited by the types of structures that modulate chelation. Suitable structures include alpha sheet, Asx turn, helix-loop-helix, beta bulge, beta hairpin, catgrip, coiled coil, collagen helix, EF hand, Greek key, helix-turn helix, leucine zipper, polyproline helix, ring finger domain, Schellman loop, ST loop, triple helix, zinc finger, or any combinations thereof. In some embodiments, the formation of protein structure brings chelation components together in the proper orientation to form a chelation moiety, but unfolding of the structure separates or misorients the chelation components. In such embodiments, switching conditions from those that favor protein (tertiary) structure formation to those that disfavor it (as well as the reverse) allows modulation of chelation efficiency.

In some embodiments, the formation of protein quaternary structure regulated formation of a chelation moiety and modulates chelation. The present invention is not limited by the types of structures that modulate chelation.

In an exemplary embodiment, a chelation construct comprises two peptides, each with a chelation component attached thereto. Under favorable conditions, each peptide forms an alpha helix, and the helices interact to form a coiled-coil domain. Formation of the coiled-coil brings the chelation components into close proximity and the proper orientation for metal ion chelation. Altering conditions to favor or disfavor formation of the coiled-coil allows modulation of chelation. Similarly, in some embodiments, a leucine zipper interaction brings chelation moieties together under favorable conditions.

In another exemplary embodiment, a chelation construct comprises one peptide with two chelation components attached thereto. Under favorable conditions, the peptide forms an alpha helix, bringing the chelation components into close proximity and the proper orientation for metal ion chelation. However, under conditions in which the alpha helix does not form, the chelation components are misaligned and do not form a chelation moiety. Altering conditions to favor or disfavor formation of the alpha helix allows modulation of chelation.

3. Peptide nucleic acid

In some embodiments, a structural moiety is a peptide nucleic acid (PNA). In some embodiments, a single chelation component is attached to a peptide nucleic acid strand. In some embodiments, two or more (e.g., 2, 3, 4, 5, 6, 7, 8, 9, 10, or more) chelation

components are attached to a peptide nucleic acid strand. In some embodiments, a peptide nucleic acid strand without an attached chelation moiety or component(s) is a structural moiety (e.g., interacting with another structural moiety or structural moieties that are attached to chelation components).

5 Chelation components are attached to peptide nucleic acid structural moieties at any suitable position or location that allows for modulation of chelation under varying conditions. In some embodiments, chelation components are attached to the peptide backbone of the peptide nucleic acid. In some embodiments, chelation components are attached to a base of the peptide nucleic acid strand (e.g., at a modified base). In some embodiments, chelation 10 components are attached at the terminus of a peptide nucleic acid strand (e.g., N-terminus or C-terminus). In some embodiments, chelation components are attached at an internal position of a peptide nucleic acid strand.

In some embodiments, one or more modified nucleotides are incorporated into a peptide nucleic acid structural moiety allow for attachment of chelation components. 15 Exemplary modified nucleotides include those with 5' acrylic phosphoramidite; 5' adenylation; 3'-, 5'-, or internal NHS ester; etc. Other suitable modified nucleotides that are used, in some embodiments, to attach chelation components to structural moieties include, but are not limited to: 4-acetylcytosine, 8-hydroxy-N6-methyladenosine, aziridinylcytosine, pseudoisocytosine, 5-(carboxyhydroxylmethyl) uracil, 5-fluorouracil, 5-bromouracil, 5- 20 carboxymethylaminomethyl-2-thiouracil, 5-carboxymethylaminomethyluracil, dihydrouracil, inosine, N6-isopentenyladenine, 1-methyladenine, 1-methylpseudouracil, 1-methylguanine, 1-methylinosine, 2,2-dimethylguanine, 2-methyladenine, 2-methylguanine, 3-methylcytosine, 5-methylcytosine, N6-methyladenine, 7-methylguanine, 5-methylaminomethyluracil, 5-methoxyaminomethyl-2-thiouracil, beta-D-mannosylqueosine, 5'- 25 methoxycarbonylmethyluracil, 5-methoxyuracil, 2-methylthio-N6-isopentenyladenine, uracil-5-oxyacetic acid methylester, uracil-5-oxyacetic acid, oxybutoxosine, pseudouracil, queosine, 2-thiocytosine, 5-methyl-2-thiouracil, 2-thiouracil, 4-thiouracil, 5-methyluracil, N-uracil-5-oxyacetic acid methylester, uracil-5-oxyacetic acid, pseudouracil, queosine, 2-thiocytosine, and 2,6-diaminopurine.

30 In some embodiments, chelation components are attached to two separate peptide nucleic acid structural moieties). Under hybridization conditions (e.g., low temperature, high salt concentration, neutral pH), the PNA structural moieties hybridize, bringing the chelation components into close proximity and the appropriate orientation to form a chelation moiety and allow metal-ion chelation. In such embodiments, chelation of metal ions (e.g., efficient

chelation of metal ions) is dependent upon the two chelation components being in close proximity and/or adopting a particular orientation. Chelator components in such embodiments are attached at locations on the separate PNA strands that render them unable to chelate metal ions (e.g., incapable of efficient chelation) when the strands are not hybridized, but capable of chelation (e.g., efficient chelation) when the strands are hybridized. In some embodiments, chelation components are located at the 5' and 3' ends of complementary strands, such that they are adjacent when the strands hybridize. In such embodiments, PNA strands may comprise chelation components at both 3' and 5' ends, such that the duplex has chelation moieties at both ends, or each stand comprises a single chelation component to produce a duplex with one chelation moiety. In other embodiments, chelation components are within the peptide nucleic acid strands such that the duplex comprises a chelation moiety internal to the strand.

In some embodiments, chelation components are attached to two separate locations on a single peptide nucleic acid structural moiety. Under hybridization and/or folding conditions (e.g., low temperature, high salt concentration, neutral pH), the peptide nucleic acid structural moiety adopts a secondary, tertiary, and/or quaternary structure that brings the chelation components into close proximity and/or into an orientation that allows for formation of a chelation moiety. The present invention is not limited by the types of PNA structures that allow for modulation of chelation.

4. Hybrid constructs

In some embodiments, a chelator construct comprises structural moieties of two different types of polymers (e.g., PNA and nucleic acid, nucleic acid and peptide, peptide and PNA, etc.). For example, in some embodiments, a PNA structural moiety is attached to a first chelation component, and a complementary nucleic acid structural moiety is attached to a second chelation component; upon hybridization of the PNA and nucleic acid, the chelation moiety is formed. In another exemplary embodiment, a peptide attached to a first chelation component and a nucleic acid, comprising a binding sequence for the peptide, attached to a second chelation component form a chelation moiety upon interaction of the peptide and nucleic acid. The present invention is not limited by the variety of hybrid chelator constructs or the mechanisms for bringing chelation components together to form chelation moieties.

5. Cross-reactivity

In some embodiments, it is important that structural moieties are not cross-reactive with other components of a reaction that the chelator construct regulates by modulating access to metal ions. For example, nucleic acid sequences are selected for chelator constructs

that will not hybridize with primer or target sequences for the amplification reactions the chelator constructs are being employed to regulate. In such embodiments, nucleic acid structural moieties have less than, for example, less than 75% sequence identity (e.g., <70%, <60%, <50%, <40%, <30%, etc.) with nucleic acid sequences (e.g., primer sequences, target sequences, non-target sequences present (or possibly present), etc.) in the reaction mixture. In some embodiments, the same cross-reactivity considerations apply to other types of reactions and other types of structural moieties (e.g., peptide, PNA, etc.). For example, peptide structural moieties are selected that do not interact with, for example, protein or nucleic acid components of a reaction mixture.

In some embodiments, structural moieties are not cross-reactive with human DNA and/or other DNA sequences being amplified in a reaction being modulated by a chelator construct.

D. Exemplary embodiments

In certain embodiments, a chelator construct is a zipper chelator. As used herein, a zipper chelator comprises two strands (e.g., separate strands or distally linked by a loop or other connection) that interact under favorable conditions through noncovalent interactions (e.g., hydrogen bonding, hydrophobic interactions, etc.) along their length to stably align the two stands (See, e.g., FIG. 3). The strands of a zipper chelator may be protein, nucleic acid, peptide nucleic acid, and/or other suitable polymers or molecular components. Suitable chelation components and structural moieties for a zipper chelator are described in sections C1-C4 above.

Under conditions that disfavor or destabilize interactions between the strands, the chelation components are separated. Conditions that favor interactions between the strands include low(er) temperature (e.g., below the T_m of the strands). In some embodiments, at sufficiently low temperature, the chelation moiety is maximally formed, resulting in the minimal K_d for the appropriate metal ions. As temperature is raised, the interaction between the strands is destabilized, and the K_d of the chelation moiety for metal ions rises. In such embodiments, the K_d of the chelation moiety is modulated by raising and lowering the temperature of the system. For example, given the appropriate ratio of chelation moieties to metal ions (e.g., excess chelation moieties), at low temperature all the available metal ions (e.g., Mg^{2+}) are sequestered; however, as the temperature of the system is raised, the K_d of the chelation moiety rises, and at a certain temperature free metal ions (e.g., Mg2+) are available in solution. By adjusting the temperature of the system, the concentration of free metal ions (e.g., Mg^{2+}) in solution is modulated.

Exemplary chelator constructs comprising three and two structural moieties are provided in FIG. 5 and FIG. 6, respectively.

E. Conditions/modulation

5 Chelation constructs are provided herein that efficiently chelate metal ions under one set of conditions, but are poor chelators under a second set of conditions. In some embodiments, altering the conditions alters the dissociation constant (K_d) for the metal ion.

In some embodiments, under metal ion sequestering conditions, a chelation construct has a K_d for a target metal ion between 1 fM and 100 μM (e.g., 1 fM… 10 fM… 100 fM… 1 10 pM… 10 pM… 100 pM… 1 mM… 10 nM… 100 nM… 1 μM… 10 μM… 100 μM). In some embodiments, under non-sequestering conditions, a chelation construct has a K_d for a target metal ion above, for example 1 μM (e.g., >1 μM… >10 μM… >100 μM… >1 mM… >10 mM… >100 mM, or more). In some embodiments, depending upon the desired application and the metal ion concentration to be used, a chelator construct with an 15 appropriate sequestering and non-sequestering K_d is designed, selected, provided, etc.

A variety of conditions may alter the favorability structure formation, hybridization, etc. of structural element(s), and therefore modulate formation of the chelator moiety. These conditions include, but are not limited to, temperature, salt concentration, solvent, pH, presence/absence/concentration of denaturants, etc.

20 In some embodiments, elevation of temperature disfavors the formation of secondary, tertiary and quaternary structures in proteins, nucleic acids, peptide nucleic acids, and hybrids thereof. Therefore, in some embodiments, a chelator construct sequesters metal ions at lower temperatures, but does not at higher temperatures. In some embodiments, the transition from sequesterer to non-sequesterer occurs at the melting temperature (T_m) of the modulating 25 structure (e.g., 30°C... 35°C... 40°C... 45°C... 50°C... 55°C... 60°C... 65°C... 70°C... 75°C... 80°C... 85°C... 90°C, or more). In some embodiments, depending upon the structure involved and the type of structural moiety, the transition may be gradual (e.g., creating a K_d gradient) or rapid (e.g., creating a steep transition from sequestering to non-sequestering).

In some embodiments, other factors and conditions, including pH, salt concentration, 30 the presence/absence of denaturants, solvent type, etc. are used to alter the T_m of structural moieties or to create an alternate set of conditions to modulate chelation (e.g., neutral vs. high pH, low vs. high salt concentration, etc.).

F. Applications

The present invention finds use in any application where metal ions in solution are desirable under one set of conditions, but undesirable under a second set of conditions. In some embodiments, the chelator constructs described herein allow modulation of metal ion concentration over the course of time (e.g., by altering the conditions).

Specific metal ions are required for the activity of many enzymes and are therefore required reagents in many assays, reactions, etc. For example, magnesium is required as a co-factor for thermostable DNA polymerase (and many other enzymes). *Taq* DNA polymerase is a magnesium-dependent enzyme and an optimum magnesium concentration is required for the success of polymerase chain reaction (PCR). However, magnesium is also a co-factor for the exonuclease activity of *Taq* DNA polymerase. Therefore, the presence of magnesium can result in degradation of the reactants (e.g., nuclease degradation of primers, substrates, and/or products) or products of a PCR reaction. Similarly, magnesium is required for the activity of RNA polymerase, but it has been demonstrated that RNA is non-specifically degraded in the presence of magnesium ions. Therefore, in both of these exemplary cases, a reversible magnesium ion chelator allows for magnesium ions to be present in solution at temperatures where DNA or RNA polymerization occur (or other enzymatic reactions), but once the temperature is reduced following the polymerization reaction, the magnesium is sequestered to prevent product degradation.

In exemplary embodiments, a nucleic acid (e.g., DNA, RNA, etc.) chelator sequesters Mg^{+2} from DNA polymerase (e.g., *Taq* DNA polymerase or proofreading *Pfu* DNA polymerase) or RNA polymerase at any temperature below the melting temperature of the secondary or tertiary structure of the nucleic acid, preventing polymerization from occurring or the reactants (e.g., nuclease degradation of primers, substrates) and/or products from being degraded (See FIG. 7A). Upon increase in temperature above the melting point, the structure of the chelator construct is altered, raising the K_d of the chelator for Mg^{2+}. Free Mg^{2+} is thereby released into solution allowing the reaction (e.g., PCR) to start (FIG. 7B). When the temperature is lowered again, the chelator structure reforms and Mg^{2+} is again unavailable (e.g., for PCR). The sequestration of Mg^{2+} prevents exonuclease activity and non-specific degradation of the products. In the absence of modulatable chelators and at low temperature, secondary polymerization products form and the polymerase exonuclease is active degrading primers and template (FIG. 7C). When the temperature is raised, in the absence of a modulatable chelator, the amplification quality is affected by accumulation of secondary products, primer dimers and decreased yield (FIG. 7D). When the temperature is lowered

again, the polymerase and exonuclease domains remain active leading to additional degradation of PCR products and reactants.

Similar condition-specific sequestration of metal ions finds use in regulating the activity of other enzymes. Chemical reactions dependent upon the presence of metal ions in solution are also regulated using chelator constructs described herein.

In other embodiments, a plurality of reversible chelation constructs is used to provide a metal-ion gradient across a volume or a surface. For example, reversible chelation constructs described herein are attached to a surface (e.g., solid support, microchannel, well, tube, etc.) or embedded within a gel or other environment (See, e.g., FIG. 4). Different portions of the surface, gel, or other environment are exposed to different conditions (e.g., structure promoting, structure disfavoring, etc.), thereby creating regions of the surface, gel, or other environment that are metal-ion chelating regions and other regions that have metal ions free in solution. Applying a gradient of conditions (e.g., temperature gradient) across the surface, gel, or other environment results in a metal-ion gradient (See, e.g., FIG. 4). In some embodiments, as enzymes and/or reactants are passed over or through the metal-ion gradient, reactions are proceed or are halted, depending upon the region conditions (e.g., structure promoting, structure disfavoring, etc.) of the environment. Such metal ion gradients may find use, for example, in NextGen sequencing applications, or other applications in which a single buffer supports multiple reaction steps (e.g., lysis, purification, ligation of adapters or other components, etc.). Other embodiments in which chelator constructs are immobilized to a surface are also contemplated.

In some embodiments, chelator constructs are used to create hot-start conditions for an enzyme. For example, a chelator construct that chelates metal ion X up to temperature Y, and an enzyme that is dependent upon metal ion X for its activity are provided in a reaction mix with the other necessary reactants. The enzyme will not catalyze the reaction until the chelator releases the metal ion X. Therefore, the reaction will only take place when the temperature of the reaction mix is brought above temperature Y. Such a setup allows for a user to apply an artificial hot-start (temperature Y) to an enzyme that would otherwise work at lower temperatures.

In some embodiments, chelator constructs are used to modulate the activity of proteases or other enzymes. For example, a chelator construct with temperature-modulatable affinity for zinc ions is used to modulate the activity of Zn-dependent proteases. The chelator construct chelates Zn ions up to temperature X; therefore, the protease that is dependent upon Zn for activity will not catalyze proteolysis at temperature X or below. Above temperature

X, the structural moiety of the chelator construct denatures or unfolds, misorienting the chelation components and releasing Zn ions into solution. Under such conditions, the protease has access to ample Zn ions and proteolysis occurs. Such a mechanism of enzyme-activity modulation is not limited to proteases and/or enzymes dependent upon Zn.
5 Modulation of other enzyme activities via selective chelation of any suitable metal ions is contemplated.

In some embodiments, chelator constructs are used to modulate copper catalyzed click chemistry reactions (*Development and Applications of Click Chemistry* Gregory C. Patton November 8, 2004; herein incorporated by reference in its entirety). The reaction requires a
10 copper catalyst and therefore only occurs under the low affinity conditions when the copper is not bound by the chelator. When the efficient chelator is present (i.e. at low temperature), there is insufficient copper concentration to catalyze the click reaction.

In some embodiments, chelator constructs are used to modulate calcium dependent signaling. Many cellular processes are responsive to extracellular calcium concentration, and
15 a reversible chelator can be used to release calcium under one set of conditions and to sequester the calcium under a second set of conditions. This could be used to provoke or inhibit a cellular response of interest.

EXPERIMENTAL

20
Example 1: Chelator compatibility with PCR

To demonstrate compatibility chelators of the present invention with PCR, a 360bp fragment of the human α-1 antitrypsin gene is amplified.

The amplifications are assembled on ice or a cold block. The magnesium chelators are titrated (0.75, 1.5, 3.0 and 4.5 mM) into reactions with the following composition: 1X
25 GoTaq® Colorless Flexi Buffer (Promega Corporation), 1.5mM $MgCl_2$, 200μM each dNTP, 1μM Forward and Reverse primer, 0.025U/μl GoTaq® DNA Polymerase (Promega Corporation), 3.3ng human genomic DNA and nuclease-free water to bring it to a 50μl reaction. No chelator control, no template control, and no primer control (to ensure that a DNA portion of the chelator, if present, does not serve as a primer) reactions are also
30 assembled. The reactions are put into a thermal cycler once the ramping for the initial denaturing cycle reaches >80°C. The following cycling protocol is used: 1 cycle (95°c for 2 minutes), 35 cycles (95°C for 15 seconds, 65°C for 30 seconds), 1 cycle (72°C for 5 minutes) and 4° soak. Once cycling is complete, PCR products are separated and visualized on a 2% agarose gel stained with ethidium bromide and UV-light illumination. A camera is used to

record the image of the gel. Template titrations (33, 3.3, 0.33, 0.033ng DNA/50µl reaction) are done to access the amount of inhibition by the chelators by looking at sensitivity.

Expected results:

1. In the no chelator control, a 360bp product is observed.
2. In reactions with a chelator that does not inhibit or is compatible with PCR, a 360bp product is observed.
3. In reactions with a chelator that is not compatible or inhibits amplification, little or no amplification product should be observed. Increasing chelator results in decreased PCR yield.
4. With the no template and no primer controls, no amplification is observed.

Example 2: Chelators provide hot-start for amplification

To demonstrate that the chelators of the present invention can provide hot-start amplification, a 1.5kb fragment of the Corynephage omega gene from plasmid DNA is amplified. If there are hot-start conditions, e.g., the magnesium is chelated at lower temperatures which inhibit DNA polymerase (e.g., *Taq* polymerase) which then is released at temperatures needed for amplification; the amplification will produce a single product that is approximately 1.5kb in size. If there are not hot-start conditions, e.g., the magnesium is not chelated at lower temperatures, and the DNA polymerase is not inhibited, the amplification will produce a product that is approximately 400bp, with possibly other secondary products, and the 1.5 kb fragment may or may not be present. To rigorously test the ability of the chelator(s) to bind magnesium and thus inhibit DNA polymerase (e.g., *Taq* DNA polymerase) activity, the amplification reactions are incubated at 22°C for six hours prior to performing PCR amplification.

The amplifications are set up at room temperature. The magnesium chelator(s) are titrated (1.25, 2.5, 5.0 and 7.5mM) into reactions with the following composition: 1X GoTaq® Colorless Flexi Buffer, 2.5mM $MgCl_2$, 200µM each dNTP, 0.4µM each primer, 0.025U/µl GoTaq® DNA Polymerase, 500pg plasmid DNA and nuclease-free water to bring it to a 25µl reaction. A "no chelator", "no template", "no primer" (to ensure that the DNA portion, if present in the chelator being tested, does not serve as a primer) and "positive hot-start" (using GoTaq® DNA polymerase with an antibody mediated hot-start) control reactions are assembled. The reactions are put in a room temperature thermal cycler, and the following cycling protocol is used: 1 cycle (22°C for 6 hours, 95°C for 2 minutes), 30 cycles (93°C for 15 seconds, 54°C for 30 seconds, 72°C for 1 minute), 1 cycle (72°C for 5 minutes), and 4°C soak. Once cycling is complete, PCR products are separated and visualized on a 1%

agarose gel with ethidium bromide staining and UV-light illumination. A camera is used to record the image of the gel.

Expected results:

1. In hot-start control (using antibody mediated hot-start DNA polymerase) and with chelator(s) exhibiting hot-start-ability, reactions produce a single 1.5kb product.
2. In chelator control and with chelator(s) not exhibiting hot-start ability, reactions produce a 400bp product. Other secondary products and the 1.5kb product may or may not be present.
3. In the "no template" and "no primer" controls, no amplification occurs.

Example 3: Thermal Reversibility

A. Evaluation of reversibility from low to high temperature and back to low temperature with stopped reactions

Activity assay reactions are assembled with and without chelator. Reactions are incubated at low temperature (e.g., 22°C or 37°C) for a period of time. Aliquots are removed, reactions stopped and samples processed. Temperatures of remainder of reactions are increased to 68-79°C, and incubated for a given amount of time. Aliquots are removed, reactions stopped and samples processed. The temperature of remainder of the reactions is lowered to the low temperature (e.g., 22°C or 37°C) and incubated for an amount of time. Aliquots are removed, reactions stopped and samples processed.

This method finds use with endpoint activity assay methods. Examples of activity assays that could be used are as follows.

1. A DNA polymerase activity assay monitoring radioactive incorporation where "activated" calf thymus or salmon sperm DNA is used as the DNA substrate. Along with DNA substrate, the reactions minimally contain a buffer (e.g., GoTaq® buffer), magnesium, dNTPs and polymerase. Reactions are stopped with EDTA, DNA precipitated by ice-cold TCA (tricholoracetic acid), incubated on ice for at least 10 minutes, filtered using GF/C filters and radioactive incorporation in precipitable DNA on filter is measured by scintillation counting. (Apospian & Kornberg. (1962) JBC 237: 519 – 525.; Chien et al. (1976) J. Bact. 127: 1550 – 1557.; herein incorporated by reference in their entireties).

2. A primer extension DNA polymerase activity assay monitoring radioactive incorporation where single-stranded DNA (e.g., M13) and primer substrate is used as the DNA substrate. The primer and template are annealed, and reactions minimally

contain buffer (e.g., GoTaq® buffer), magnesium, dNTPs and polymerase. Reactions are stopped with EDTA, DNA precipitated by ice-cold TCA, incubated on ice for at least 10 minutes, filtered using GF/C filters and radioactive incorporation in precipitable DNA on filter is measured by scintillation counting. (Longley & Mosbaugh. (1991) Biochemistry 30: 2655 – 2664.; herein incorporated by reference in its entirety).

3. A 5' nuclease assay (for an enzyme such as *Taq*) where a 5' fluorescently dye-labeled bifurcated duplex DNA substrate is used (Lyamichev et al. (1993) Science 260: 778-783.; Lyamichev (1999) PNAS 96:6143-6148.; Ceska & Sayers (1998) TIBS: 331-336.; herein incorporated by reference in their entireties). The DNA substrate is annealed and combined with reaction components including buffer (e.g., GoTaq® buffer), magnesium and nuclease or polymerase with nuclease domain. Reactions are stopped with EDTA and run on a capillary electrophoresis instrument to determine amount of cut and uncut DNA substrate.
4. A 3' to 5' exonuclease (for a proofreading polymerase such as *Pfu*) assay where 3'-radiolabeled duplexed DNA is used as a substrate. The DNA substrate is combined with reaction components including buffer (e.g., GoTaq® buffer), magnesium and nuclease or polymerase with nuclease domain. Reactions are stopped by EDTA, DNA precipitated by ice-cold TCA and incubated on ice for at least 10 minutes. Precipitable DNA is pelleted by centrifugation and released non-precipitable DNA from radioactively labeled 3' end is measured by scintillation counting (Chase & Richardson. (1974) JBC 249: 4545 – 4552.; Sambrook et al. (1989) Molecular Cloning: A Laboratory Manual (2nd Edition). Pages 10.51-52.; herein incorporated by reference in their entireties).

Expected results:

1. During the first low temperature incubation, the chelator and cation are bound. For reactions with chelator, little or no activity/product accumulation is expected. For reactions without chelator, activity/product accumulation characteristic of low temperature incubation is expected.
2. During the high temperature incubation, the cation and chelator are not bound. For reactions with and without chelator, a high amount of activity/product accumulation is expected. The amount of activity/product accumulation should be similar for reactions with and without chelator.

3. During the second low temperature incubation, the chelator and cation are bound again. For reactions with chelator, little or no activity/product accumulation is expected. For reactions without chelator, activity/ product accumulation characteristic of low temperature incubation is expected.

5 B: Evaluation of reversibility from low to high temperatures and back to low temperature with a real-time extension rate assays

This method is similar to A except it uses a real-time extension rate activity assay method to measure nucleotide incorporation of a DNA polymerase. An example of this would be a primer extension assay where extension is monitored on a real-time PCR
10 instrument using noncovalent DNA dyes such as BRYT™ Green or SYBR® Green and oligonucleotide DNA substrate (Montgomery & Wittwer. (2014) Clinical Chemistry 60(2):334-340).; herein incorporated by reference in its entirety). Activity assay reactions are assembled with and without chelator. The reactions minimally include buffer (e.g., GoTaq® buffer), magnesium, dNTPs, DNA substrate and polymerase. The reactions are incubated at
15 low temperature (e.g., 22°C or 37°C) for a given period of time measuring extension rate. Temperatures of reactions are then increased to 68-79°C and incubated for a given amount of time measuring extension rate. Temperature of the reactions are then lowered to the low temperature (e.g., 22°C or 37°C) and incubated for a given amount of time measuring extension rate.

20 Expected results:

1. During the first low temperature incubation, the chelator and cation are bound. For reactions with chelator, low or no activity/extension rate is expected. For reactions without chelator, activity/extension rate characteristic of low temperature incubation is expected.
2. During the high temperature incubation, the cation and chelator are not bound. For reactions with and without chelator, high activity/extension rate is expected. The extension rate is similar for reactions with and without chelator. (25)
3. During the second low temperature incubation, the chelator and cation are bound again. For reactions with chelator, low or no activity/extension rate is expected. For
30 reactions without chelator, activity/extension rate characteristic of low temperature incubation is expected.

C: Evaluation of reversibility, ruggedness and stability of chelator in PCR

The 5' nuclease activity of *Taq* DNA polymerase at low temperature is used to monitor chelator and cation binding before and after amplification reactions. The

amplification monitors the availability of magnesium at higher temperatures. Amplification reactions are assembled with and without chelators. Reactions include minimally buffer (e.g., GoTaq® buffer), magnesium, dNTPs, primers, plasmid template and polymerase. Hot-start PCR amplification of Corynephage omega gene could be used.

- For set 1 of the reactions, 5' fluorescently dye-labeled bifurcated duplex DNA substrate (see A, assay example 3) is added to reactions. Reaction is incubated at room temperature or 4°C for a period of time. Reactions are stopped with EDTA and analyzed by capillary electrophoresis to determine the amount of cut and uncut DNA substrate.
- For set 2 of the reactions, reactions are incubated at room temperature or 4°C for period of time in parallel with set 1. Reactions are then incubated in a thermal cycler using the following cycling program [1 cycle (22°C for 6 hours, 95°C for 2 minutes), 30 cycles (93°C for 15 seconds, 54°C for 30 seconds, 72°C for 1 minute), 1 cycle (72°C for 5 minutes), and 4°C soak]. Separate and visualize PCR products on a 1% agarose gel stained with ethidium bromide and UV-light. A camera is used to record the gel image.
- For set 3, reactions are incubated at room temperature or 4°C for period of time then amplified as done with set 2 (reactions done in parallel). Next, 5' fluorescently dye-labeled bifurcated duplex DNA substrate is added and reactions are incubated at room temperature or 4°C for period of time. Reactions are stopped with EDTA and analyzed by capillary electrophoresis to determine the amount of cut and uncut DNA substrate.

Expected results:

1. With chelator (if reversible)
    - For the first set of reactions incubated at low temperature, no cutting of 5' labeled DNA substrate is expected. The 5' nuclease activity of *Taq* DNA polymerase is inhibited.
    - For the second set of reactions incubated at low temperature then amplified, only a 1.5kb product in hot-start amplification is expected. The 400bp or secondary PCR products is not observed, since magnesium is not available at low temperature, but is available at high temperature making amplification possible.
    - For the third set of reactions subjected to low temperature, amplification and then the second low temperature incubation, no cutting of 5' labeled DNA substrate is

expected. The 5' nuclease activity of *Taq* DNA polymerase is inhibited again. If the chelator does not survive PCR intact, then the 5' labeled DNA substrate will be cut.

2. With chelator (if not reversible)
    - For the first set of reactions incubated at low temperature, no cutting of 5' labeled DNA substrate is expected. The 5' nuclease activity of Taq DNA polymerase is inhibited.
    - For the second set of reactions incubated at low temperature then amplified, no amplification is expected.
    - For the third set of reactions subjected to low temperature, amplification and then the second low temperature incubation, no cutting of the 5' labeled DNA substrate is expected.

3. Without chelator
    - For the first set of reactions incubated at low temperature, the 5' labeled DNA substrate is expected to be cut at levels appropriate for that temperature. The 5' nuclease activity of *Taq* DNA polymerase is not inhibited.
    - For the second set of reactions incubated at low temperature then amplified, the 400bp product, indicating no hot-start amplification since magnesium is available at low and high temperature, is expected. Other secondary products may be observed.
    - For the third set of reactions subjected low temperature incubation, amplification and then the second low temperature incubation, the 5' labeled DNA substrate is expected to be cut. The 5' nuclease activity of *Taq* DNA polymerase is not inhibited

D: Hot-Start model PCR reversibility

The amplification experiment above to demonstrate that the chelator(s) allow hot-start amplification also demonstrates reversible binding of the chelator and cation. At low temperature, if the chelator and magnesium are bound, the polymerase cannot generate spurious products since there is no available magnesium. Only a single 1.5kb PCR product is made. At high temperature, if the magnesium is free, then amplification products will be generated. If the magnesium and chelator were not dissociated, then products would not be formed since reaction requires magnesium.

E: Reversibility and easy visualization of temperatures where chelator and magnesium are or are not bound.

Restriction enzyme digests are assembled with DNA substrate containing a restriction enzyme cut-site and using enzymes that have activity over a broad temperature range, e.g., BstXI and BssHII. BstXI and BssHII have temperature optima at 50°C, but retain 75-100% of their activity at 37°C and are not or are only slightly heat inactivated at 65°C. These enzymes also require magnesium. Reactions include the specific restriction endonuclease buffer, magnesium, DNA substrate and enzyme. Reactions are stopped by the addition of EDTA.

The restriction enzyme digests are tested and compared with and without chelator at different temperatures and incubation times. The restriction digest products are separated and visualized on an agarose gel stained with ethidium bromide and UV-light illumination. A camera is used to record image of gel. Accumulation or lack of accumulation of cleavage product is observed.

Expected results:

1. If the chelator binds magnesium at lower temperatures, there is no cutting of the DNA substrate. Without a chelator, there is cleavage of the DNA substrate.
2. As the temperature is increased and the chelator and magnesium start to dissociate, there is cleavage in the reactions containing chelator, and there cleavage continues in reactions without chelator.
3. When the temperature is high enough, the chelator and magnesium are completely dissociated, and reactions with and without chelator exhibit similar levels of cleavage.
4. To show reversibility from high to low temperature, the reactions can be switched to a low temperature. Reactions with chelator will stop accumulating cleaved substrate, but reactions without chelator will continue to accumulate product.

All publications and patents mentioned in the present application are herein incorporated by reference in their entireties. Various modification and variation of the described methods and compositions of the invention will be apparent to those skilled in the art without departing from the scope and spirit of the invention. Although the invention has been described in connection with specific preferred embodiments, it should be understood that the invention as claimed should not be unduly limited to such specific embodiments. Indeed, various modifications of the described modes for carrying out the invention that are obvious to those skilled in the relevant fields are intended to be within the scope of the following claims.

CLAIMS

1. A reversible chelator construct comprising one or more structural moieties attached to two or more chelation components, wherein, under high temperature conditions, said one or more structural moieties adopt a first conformation that positions and/or orients said two or more chelation components such that said two or more chelation components less efficiently chelate metal ions, and wherein under low temperature conditions said one or more structural moieties adopt a second conformation that positions and/or orients said two or more chelation components such that said two or more chelation components more efficiently chelate metal ions.

2. The reversible chelator construct of claim 1, wherein said one or more structural moieties are selected from peptides, nucleic acids, and peptide nucleic acids.

3. The reversible chelator construct of claim 2, wherein said one or more structural moieties are nucleic acids.

4. The reversible chelator construct of claim 3, comprising a single stem-loop forming nucleic acid strand and two chelation components, wherein formation of the stem-loop brings the chelation components into appropriate proximity and orientation to efficiently chelate metal ions, and wherein melting of the stem loop separates the chelation components such that said chelation components are unable to efficiently chelate metal ions.

5. The reversible chelator construct of claim 4, wherein the chelation components are attached to 5' and 3' termini of the nucleic acid strand.

6. The reversible chelator construct of claim 4, wherein the chelation components are attached to the nucleic acid strand internally.

7. The reversible chelator construct of claim 3, comprising first nucleic acid strand attached to a first chelation component and a second nucleic acid strand attached to a second chelation component, wherein the first and second nucleic acid strands are complementary, wherein hybridization of the nucleic acid strands brings the chelation components into appropriate proximity and orientation to efficiently chelate metal ions, and wherein melting

of the nucleic acid strands separates the chelation components such that said chelation components are unable to efficiently chelate metal ions.

8. The reversible chelator construct of claim 7, wherein the first chelation component is attached to the 5' end of the first nucleic acid strand and the second chelation component is attached to the 3' end of the second nucleic acid strand.

9. The reversible chelator construct of claim 7, wherein the chelation components are attached to the nucleic acid strands internally.

10. The reversible chelator construct of claim 3, comprising a nucleic acid strand not attached to a chelation component and one or more nucleic acid strands attached chelation components, wherein hybridization of the one or more nucleic acid strands attached chelation components to nucleic acid strand not attached to a chelation component brings the chelation components into appropriate proximity and orientation to efficiently chelate metal ions, and wherein melting of the nucleic acid strands separates the chelation components such that said chelation components are unable to efficiently chelate metal ions.

11. The reversible chelator construct of claim 1, wherein the two or more chelation components are iminodiacetic acid moieties.

12. The reversible chelator construct of claim 1, wherein high temperature is a temperature above a transition temperature between the first and second confirmations.

13. The reversible chelator construct of claim 1, wherein low temperature is a temperature below a transition temperature between the first and second confirmations.

14. The reversible chelator construct of claim 1, wherein less efficient chelation is a K_d of 10 μM or more.

15. The reversible chelator construct of claim 14, wherein less efficient chelation is a K_d of 100 μM or more.

16. The reversible chelator construct of claim 15, wherein less efficient chelation is a K_d of 1 mM or more.

17. The reversible chelator construct of claim 1, wherein less efficient chelation is a K_d of 1 μM or less.

18. The reversible chelator construct of claim 17, wherein less efficient chelation is a K_d of 100 nM or less.

19. The reversible chelator construct of claim 18, wherein less efficient chelation is a K_d of 10 nM or less.

20. A method of regulating an enzymatic reaction comprising:

(a) providing in a reaction mix:

(i) an enzyme that catalyzes said enzymatic reaction, wherein said enzyme requires a metal ion cofactor for catalysis;

(ii) a substrate for said enzyme;

(iii) optionally any other reactants for said enzymatic reaction;

(iv) the metal ion cofactor for said enzyme; and

(v) a reversible chelator construct that is incapable of efficiently chelating said metal ion cofactor under a first set of conditions and efficiently chelates said metal ion cofactor under a second set of conditions;

(b) exposing said reaction mix to said first set of conditions, wherein said enzyme inefficiently catalyzes said enzymatic reaction under said first set of conditions because said metal ion cofactor is chelated by the reversible chelator construct; and

(c) exposing said reaction mix to said second set of conditions, wherein said enzyme efficiently catalyzes said enzymatic reaction under said second set of conditions because said metal ion cofactor is available in solution.

21. The method of claim 20, wherein step (b) is performed before step (c).

22. The method of claim 21, wherein further comprising:

(d) repeating step (b).

23. The method of claim 20, wherein step (c) is performed before step (b).

24. The method of claim 23, wherein further comprising:
(d) repeating step (c).

25. The method of claim 20, wherein said enzyme is DNA polymerase said metal ion cofactor is magnesium.

26. The method of claim 25, wherein said enzymatic reaction is polymerase chain reaction.

27. The method of claim 25, wherein said enzymatic reaction is 3' to 5' cleavage of nucleic acids.

28. The method of claim 25, wherein said enzymatic reaction is 5' to 3' cleavage of nucleic acids.

29. The method of claim 20, wherein inefficient catalysis is less than 50% enzyme activity.

30. The method of claim 29, wherein inefficient catalysis is less than 10% enzyme activity.

31. The method of claim 30, wherein inefficient catalysis is less than 1% enzyme activity.

32. The method of claim 20, wherein efficient catalysis is greater than 50% enzyme activity.

33. The method of claim 32, wherein efficient catalysis is greater than 75% enzyme activity.

34. The method of claim 33, wherein efficient catalysis is greater than 90% enzyme activity.

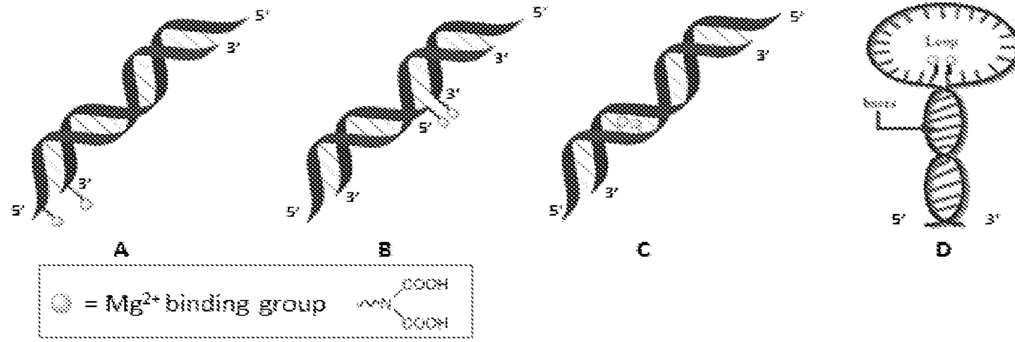
35. A composition comprising one or more biopolymers and two or more iminodiacetic acid moieties, wherein upon folding and/or hybridization of said one or more biopolymers said two or more iminodiacetic acid moieties are positioned adjacent to one another.

36. The composition of claim 35, wherein said one or more biopolymers are selected from nucleic acids, peptides, and peptide nucleic acids.

37. The composition of claim 35, wherein said two or more iminodiacetic acid moieties are attached to the same biopolymer of said one or more biopolymers.

38. The composition of claim 35, wherein said two or more iminodiacetic acid moieties are attached to the separate biopolymers of said one or more biopolymers.

FIG. 1



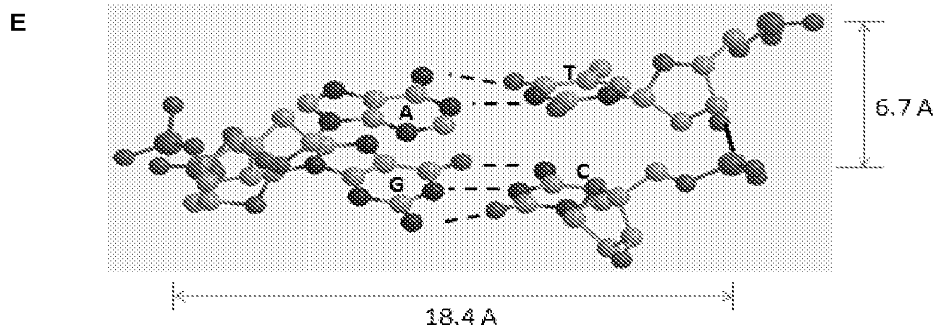


FIG. 2A

FIG. 2B

FIG. 2C

FIG. 2D

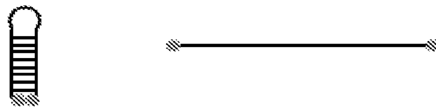


FIG. 2E

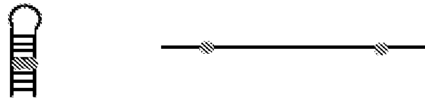


FIG. 2F

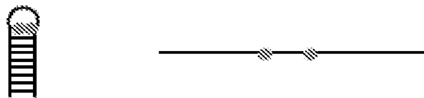


FIG. 2G

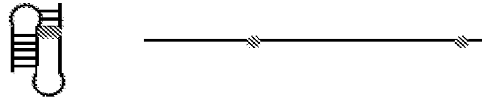


FIG. 2H

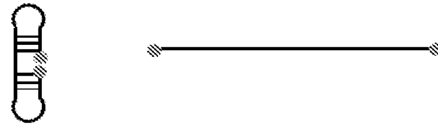
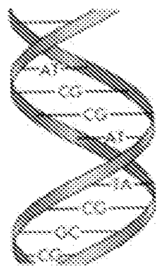


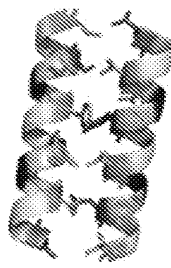
FIG. 2I

3/9

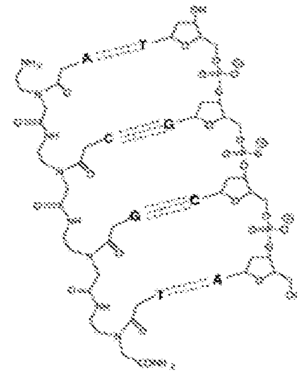
FIG. 3



DNA



Coiled Coil



PNA-DNA

FIG. 4

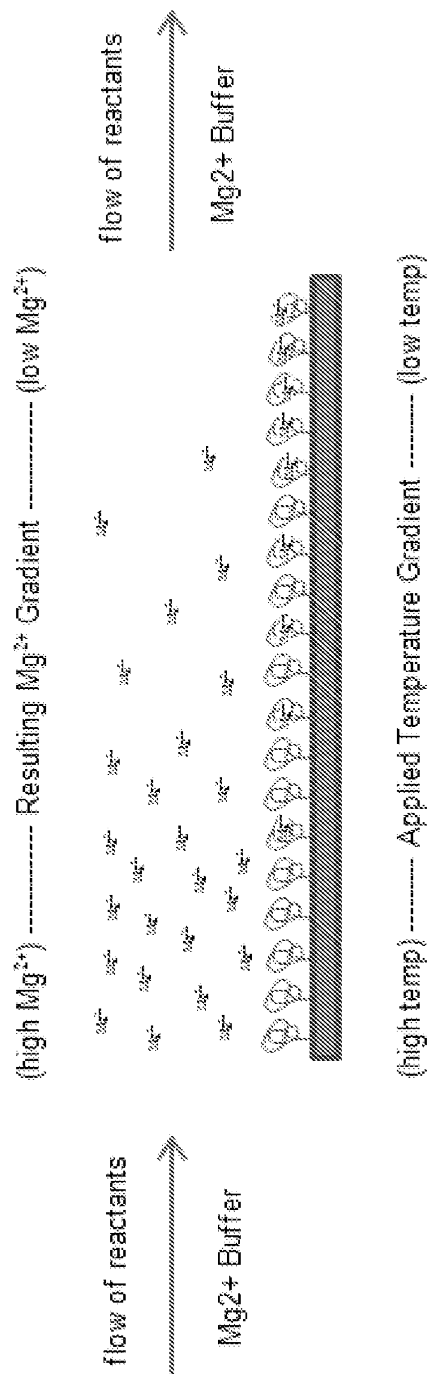
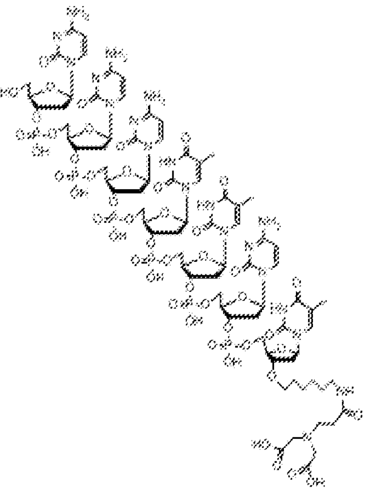


FIG. 5

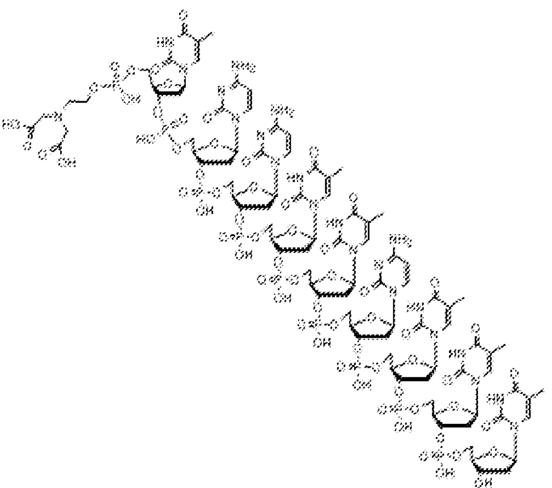
Molecule 1: 5'-C-C-C-T-T-C-T(di-acid)-3' (SEQ ID NO: 1)

Molecule 2: 5'-(di-acid)T-C-C-T-T-C-T-T-T-3' (SEQ ID NO: 2)

Molecule 3: 5'-A-A-A-G-A-A-G-G-A-A-G-A-A-G-G-G-3' (SEQ ID NO: 3)



Molecule 1



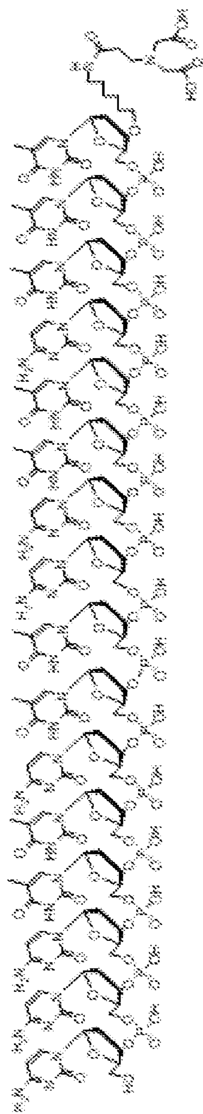
molecule 2

Molecule 3

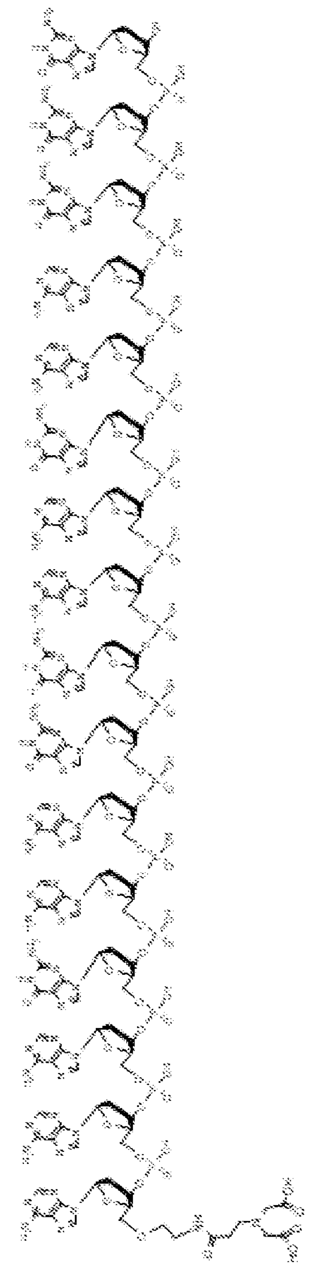
FIG. 6

Molecule 1: 5'-C-C-C-T-T-C-T- T-C-C-T-T-C-T-T-T(di-acid)-3'(SEQ ID NO: 4)

Molecule 2: 5'-(di-acid)A-A-G-A-A-G-G-A-A-G-A-A-G-G-G-3' (SEQ ID NO: 5)



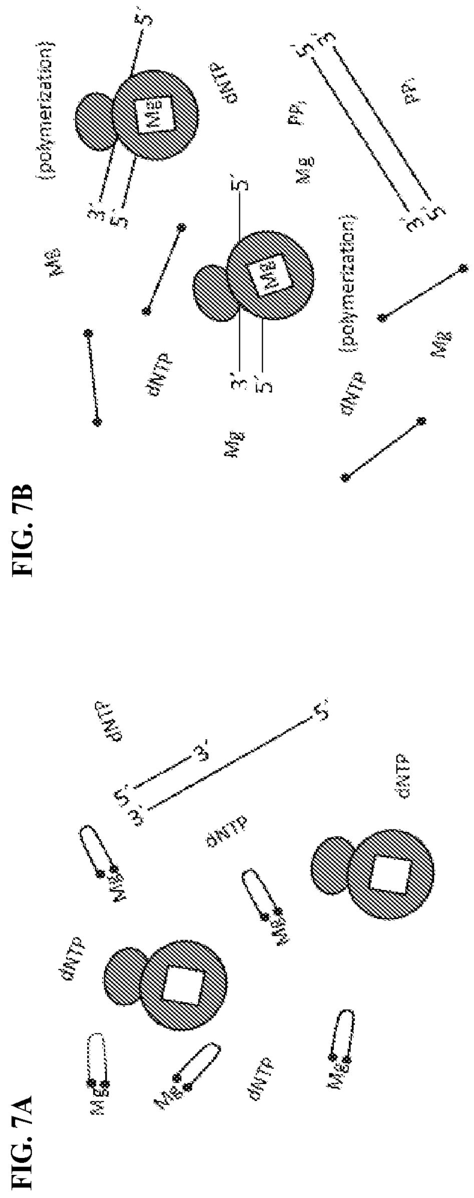
Molecule 1

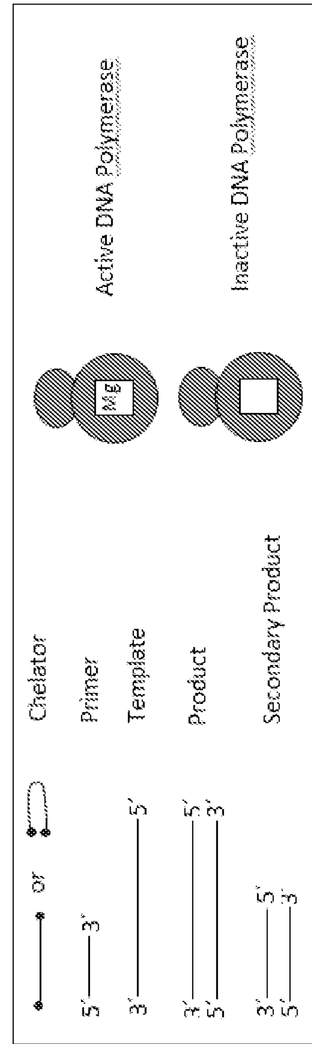


Molecule 2

FIG. 7A

FIG. 7B





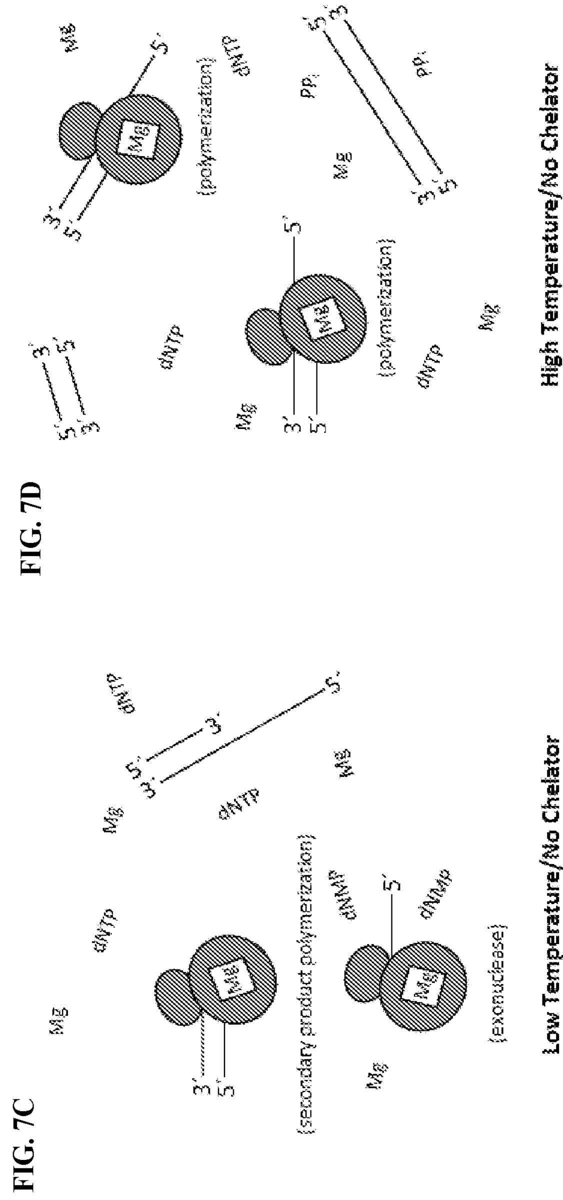


FIG. 7C
Mg
dNTP
dNTP
Mg
(secondary product polymerization)
dNMP
dNMP
(exonuclease)
Low Temperature/No Chelator
FIG. 7D
dNTP
(polymerization)
dNTP
Mg
Mg
PPi
PPi
High Temperature/No Chelator

(12) INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(19) World Intellectual Property Organization
International Bureau

(43) International Publication Date
7 January 2016 (07.01.2016)

(10) International Publication Number
WO 2016/004333 A3

(51) International Patent Classification:
C12Q 1/68 (2006.01) *G01N 33/00* (2006.01)
G01N 33/53 (2006.01) *C40B 30/04* (2006.01)

(21) International Application Number: PCT/US2015/039036

(22) International Filing Date: 2 July 2015 (02.07.2015)

(25) Filing Language: English

(26) Publication Language: English

(30) Priority Data:
62/020,227 2 July 2014 (02.07.2014) US

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(81) Designated States *(unless otherwise indicated, for every kind of national protection available)*: AE, AG, AL, AM, AO, AT, AU, AZ, BA, BB, BG, BH, BN, BR, BW, BY, BZ, CA, CH, CL, CN, CO, CR, CU, CZ, DE, DK, DM, DO, DZ, EC, EE, EG, ES, FI, GB, GD, GE, GH, GM, GT, HN, HR, HU, ID, IL, IN, IR, IS, JP, KE, KG, KN, KP, KR, KZ, LA, LC, LK, LR, LS, LU, LY, MA, MD, ME, MG, MK, MN, MW, MX, MY, MZ, NA, NG, NI, NO, NZ, OM, PA, PE, PG, PH, PL, PT, QA, RO, RS, RU, RW, SA, SC, SD, SE, SG, SK, SL, SM, ST, SV, SY, TH, TJ, TM, TN, TR, TT, TZ, UA, UG, US, UZ, VC, VN, ZA, ZM, ZW.

(84) Designated States *(unless otherwise indicated, for every kind of regional protection available)*: ARIPO (BW, GH, GM, KE, LR, LS, MW, MZ, NA, RW, SD, SL, ST, SZ, TZ, UG, ZM, ZW), Eurasian (AM, AZ, BY, KG, KZ, RU, TJ, TM), European (AL, AT, BE, BG, CH, CY, CZ, DE, DK, EE, ES, FI, FR, GB, GR, HR, HU, IE, IS, IT, LT, LU, LV, MC, MK, MT, NL, NO, PL, PT, RO, RS, SE, SI, SK, SM, TR), OAPI (BF, BJ, CF, CG, CI, CM, GA, GN, GQ, GW, KM, ML, MR, NE, SN, TD, TG).

Published:
— *with international search report (Art. 21(3))*
— *before the expiration of the time limit for amending the claims and to be republished in the event of receipt of amendments (Rule 48.2(h))*
— *with sequence listing part of description (Rule 5.2(a))*

(88) Date of publication of the international search report: 24 March 2016

WO 2016/004333 A3

(54) Title: REVERSIBLE METAL ION CHELATORS

(57) Abstract: Provided herein are chelator constructs (e.g., nucleic acid, peptide, peptide nucleic acid, etc.) that sequester metal ions (e.g., Mg^{2+}) under a first set of conditions and fail to sequester or release sequestered metal ions under a second set of conditions. In particular, nucleic acid constructs are provided that sequester metal ions (e.g., Mg^{2+}) under conditions that favor secondary and tertiary structure formation and release or fail to sequester metal ions under conditions that disfavor the formation of such structures.

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US 15/39036

A. CLASSIFICATION OF SUBJECT MATTER
IPC(8) - C12Q 1/68; G01N 33/53, 33/00; C40B 30/04 (2015.01)
CPC - C12Q 1/6825; G01N 33/52, 33/53, 33/68; B01J 2219/00585
According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC(8) - C12Q 1/68; G01N 33/53, 33/00; C40B 30/04 (2015.01)
CPC - C12Q 1/6825; G01N 33/52, 33/53, 33/68; B01J 2219/00585

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched
CPC - C12Q 1/6825; G01N 33/52, 33/53, 33/68; B01J 2219/00585 (text search)
USPC: 435/6.1, 7.1; 436/94, 86; 506/9 (text search)

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)
Electronic data bases: PatBase; Google Patents; Google Scholar
Search terms: Conformationally contrainted chelator construct (bidentate), closed or open position, structural element (e.g. peptide, DNA, RNA, PNA), reversible chelation, chelation components (e.g. terpyridine (TPY), iminodiacetic acid (IDA))

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X --- Y	MORGAN et al. Reversible metal-dependent destabilization and stabilization of a stem-chelate-loop probe binding to an unmodified DNA target. Bioconjug Chem 17 October 2012 Che Vol 23 No 10 Pages 2020-2024. Especially pg 2020 fig 1, pg 2021 Table 1.	1, 12, 13 ------------- 2, 11, 14-19
Y	US 2008/0269065 A1 (LYON et al.) 30 October 2008 (30.10.2008). Especially para [0023], [0024], [0025], [0033], [0034], [0087].	2, 11, 14-19

☐ Further documents are listed in the continuation of Box C. ☐

* Special categories of cited documents:
"A" document defining the general state of the art which is not considered to be of particular relevance
"E" earlier application or patent but published on or after the international filing date
"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)
"O" document referring to an oral disclosure, use, exhibition or other means
"P" document published prior to the international filing date but later than the priority date claimed
"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
26 October 2015 (26.10.2015)	12 JAN 2016

Name and mailing address of the ISA/US	Authorized officer:
Mail Stop PCT, Attn: ISA/US, Commissioner for Patents P.O. Box 1450, Alexandria, Virginia 22313-1450 Facsimile No. 571-273-8300	Lee W. Young PCT Helpdesk: 571-272-4300 PCT OSP: 571-272-7774

Form PCT/ISA/210 (second sheet) (January 2015)

INTERNATIONAL SEARCH REPORT

International application No. PCT/US 15/39036

Box No. II Observations where certain claims were found unsearchable (Continuation of item 2 of first sheet)

This international search report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☐ Claims Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:

2. ☐ Claims Nos.:
because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:

3. ☐ Claims Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box No. III Observations where unity of invention is lacking (Continuation of item 3 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:
------Go to Extra Sheet for continuation------

1. ☐ As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims.

2. ☐ As all searchable claims could be searched without effort justifying additional fees, this Authority did not invite payment of additional fees.

3. ☐ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims for which fees were paid, specifically claims Nos.:

4. ☒ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:
Claims 1-19, limited to the first named structural moiety, peptides (claim 2) (Claims 1, 2, 11-19)

Remark on Protest

☐ The additional search fees were accompanied by the applicant's protest and, where applicable, the payment of a protest fee.

☐ The additional search fees were accompanied by the applicant's protest but the applicable protest fee was not paid within the time limit specified in the invitation.

☐ No protest accompanied the payment of additional search fees.

Form PCT/ISA/210 (continuation of first sheet (2)) (January 2015)

----continuation of Box III (Lack of Unity of Inventon)------

This application contains the following inventions or groups of inventions which are not so linked as to form a single general inventive concept under PCT Rule 13.1: In order for all inventions to be examined, the appropriate additional examination fees must be paid.

Group I+: Claims 1-19, directed to a reversible chelator construct comprising one or more structural moieties attached to two or more chelation components. The reversible chelator construct will be searched to the extent that the one or more structural moieties encompasses the first named structural moiety, peptides. It is believed that claims 1-2, 11-19 encompass this first named invention, and thus these claims will be searched without fee to the extent that they encompass peptides. Additional structural moieties will be searched upon the payment of additional fees. Applicants must specify the claims that encompass any additionally elected structural moieties. Applicants must further indicate, if applicable, the claims which encompass the first named invention, if different than what was indicated above for this group. Failure to clearly identify how any paid additional invention fees are to be applied to the "+" group(s) will result in only the first claimed invention to be searched. An exemplary election of structural moieties would be nucleic acids (claims 1-19).

Group II: claims 20-34, drawn to a method of regulating an enzymatic reaction.

Group III: claims 35-38, drawn to a composition comprising one or more biopolymers and two or more iminodiacetic acid moieties
The inventions listed as Groups I+, II and III do not relate to a single general inventive concept under PCT Rule 13.1 because, under PCT Rule 13.2, they lack the same or corresponding special technical features for the following reasons:

Special Technical Features

Group I+ includes the special technical feature of a reversible chelator construct wherein said construct adopts different conformations at varying temperatures, not required by Groups II and III.

Group II includes the special technical feature of a method of regulating an enzymatic reaction by regulating the availability of metal ion cofactor in solution, not required by Groups I+ and III.

Group III includes the special technical feature of folding and/or hybridization of one or more biopolymers, not required by Groups I+ and III.

Another special technical feature of the inventions listed as Group I+ is the specific structural moieties, recited therein. Each of the inventions of Group I+ requires a unique structural moiety, not required by the other inventions

Common Technical Features

1. The inventions of Groups I+, II and III share the technical feature of a chelator construct.

2. The inventions of Groups I+ and II share the technical feature of a reversible chelator construct, that is incapable of efficiently chelating said metal ion cofactor under a first set of conditions and efficiently chelates said metal ion cofactor under a second set of condition.

3. The inventions of Groups I+ and III share the technical feature of a composition comprising structural moieties attached to two or more chelation components and upon changes in the structural moieties, said two or more chelation components are positioned adjacent to one another.

However, said common technical features do not represent a contribution over the prior art, and are obvious over the publication titled "Reversible metal-dependent destabilization and stabilization of a stem-chelate-loop probe binding to an unmodified DNA target" by MORGAN et al. (hereinafter "Morgan") [published 17 October 2012 in Bioconjug Chem Vol 23 No 10 Pages 2020-2024].

Concerning common technical feature #1, Morgan teaches a chelator construct (see pg 13 Table 1; one oligonucleotide (structural moiety) with two chelation components (e.g. TPY = terpyridine); appropriately modified oligos illustrated are compound 4 and compound 6).

Concerning common technical feature #2, a reversible chelator construct that is incapable of efficiently chelating said metal ion cofactor under a first set of conditions and efficiently chelates said metal ion cofactor under a second set of condition, , it would have been obvious, based on Morgan figure 1 (pg 7 fig 1; left structure in figure), and knowledge of stability of stem-loop structures as a function of temperature, as was well known in the art, that increasing the solution temperature above the Tm of the stem-loop structure would have melted the stem loop structure and converted it into a random structure, whereby that the two chelation components could no longer adopt a conformation in which they were close to one another and more efficiently chelate metal ions, as compared to at a temperature below the Tm of the stem-loop.

----go to next sheet for continuation---------

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Concerning common technical feature #3, structural moieties attached to two or more chelation components and upon changes in the structural moieties, said two or more chelation components are positioned adjacent to one another, Morgan teaches structural moieties attached to two or more chelation components (pg 13 Table 1; one oligonucleotide (structural moiety) with two chelation components (e.g. TPY =terpyridine); appropriately modified oligos illustrated are compound 4 and compound 6). Furthermore, it would have been obvious to an artisan of ordinary skill in the art that adjusting the temperature above or below the Tm of the stem loop structure in Morgan Fig 1 (pg 7, left structure in figure) would have regulated the conformation of the stem loop structure as melting into a random structure (above Tm), or allowing formation of a stable stem loop structure (below Tm). It would have been obvious that lowering the temperature below Tm, would, as a result in upon changes in the structural moieties (to form stem loop, below Tm), that said two or more chelation components are positioned adjacent to one another (as depicted in pg 7 fig 1 left structure).

Concerning Group I+, claim 1, Morgan teaches a reversible chelator construct comprising one or more structural moieties attached to two or more chelation components (see pg 13 Table 1; one oligonucleotide (structural moiety) with two chelation components (e.g. TPY =terpyridine); appropriately modified oligos illustrated are compound 4 and compound 6). Morgan does not specifically teach under high temperature conditions, said one or more structural moieties adopt a first conformation that positions and/or orients said two or more chelation components such that said two or more chelation components less efficiently chelate metal ions, and wherein under low temperature conditions said one or more structural moieties adopt a second conformation that positions and/or orients said two or more chelation components such that said two or more chelation components more efficiently chelate metal ions. However, it would have been obvious, based on Morgan figure 1 (pg 7 fig 1, left structure in figure), and knowledge of stability of stem-loop structures as a function of temperature, as was well known in the art, that increasing the solution temperature beyond the Tm of the stem-loop structure would have melted the stem loop structure and converted it into a random structure, whereby that the two chelation components could no longer adopt a conformation in which they were close to one another and more efficiently chelate metal ions, as compared to at a temperature below the Tm of the stem-loop.

As the common technical features were known in the art at the time of the invention, they cannot be considered common special technical features that would otherwise unify the groups. The inventions lack unity with one another.

Therefore, Groups I+, II and III lack unity of invention under PCT Rule 13 because they do not share a same or corresponding special technical feature

(12) INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(19) World Intellectual Property Organization
International Bureau

(43) International Publication Date
13 February 2003 (13.02.2003)

PCT

(10) International Publication Number
WO 03/012066 A2

(51) International Patent Classification⁷: C12N

(21) International Application Number: PCT/US02/24533

(22) International Filing Date: 2 August 2002 (02.08.2002)

(25) Filing Language: English

(26) Publication Language: English

(30) Priority Data:
60/309,646 2 August 2001 (02.08.2001) US
09/920,872 2 August 2001 (02.08.2001) US
10/091,784 6 March 2002 (06.03.2002) US

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(81) Designated States *(national)*: AE, AG, AL, AM, AT, AU, AZ, BA, BB, BG, BR, BY, BZ, CA, CH, CN, CO, CR, CU, CZ, DE, DK, DM, DZ, EC, EE, ES, FI, GB, GD, GE, GH, GM, HR, HU, ID, IL, IN, IS, JP, KE, KG, KP, KR, KZ, LC, LK, LR, LS, LT, LU, LV, MA, MD, MG, MK, MN, MW, MX, MZ, NO, NZ, OM, PH, PL, PT, RO, RU, SD, SE, SG, SI, SK, SL, TJ, TM, TN, TR, TT, TZ, UA, UG, US, UZ, VN, YU, ZA, ZM, ZW.

(84) Designated States *(regional)*: ARIPO patent (GH, GM, KE, LS, MW, MZ, SD, SL, SZ, TZ, UG, ZM, ZW), Eurasian patent (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European patent (AT, BE, BG, CH, CY, CZ, DE, DK, EE, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE, SK, TR), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, GQ, GW, ML, MR, NE, SN, TD, TG).

Published:
— *without international search report and to be republished upon receipt of that report*

For two-letter codes and other abbreviations, refer to the "Guidance Notes on Codes and Abbreviations" appearing at the beginning of each regular issue of the PCT Gazette.

WO 03/012066 A2

(54) **Title:** MAGNESIUM PRECIPITATE HOT START METHOD FOR MOLECULAR MANIPULATION OF NUCLEIC ACIDS

(57) **Abstract:** The present invention provides methods of performing enzymatic reactions, including PCR, which require the use of magnesium dependent enzymes, including restriction endonucleases, ligases, and reverse transcriptases. The method is based on sequestration of magnesium ions in the form of a precipitate which renders a magnesium dependent enzyme inactive until the appropriate time in the reaction when a certain temperature is reached and the magnesium ions are released from the precipitate. Also provided are kits comprising reagents and instructions for amplifying a target nucleic acid, for DNA digestion and ligation, and for reverse transcription ofRNA into cDNA. Furthermore, the kits and reagents of the present invention can be utilized in other reactions requiring magnesium dependent enzymes.

5 # MAGNESIUM PRECIPITATE HOT START METHOD FOR MOLECULAR MANIPULATION OF NUCLEIC ACIDS

CROSS-REFERENCE TO RELATED APPLICATIONS

This application claims the benefit and priority of U.S. provisional application
10 serial no. 60/309,646, filed August 2, 2001 as well as U.S. non-provisional application serial no. 10/091,784 filed March 6, 2002 and its parent, U.S. non-provisional application serial no. 09/920,872, filed August 2, 2001 and issued June 11, 2002 as U.S. Patent No. 6,403,341, all the specifications of which are included herein by reference as if restated here in full.

15 ## Field of the Invention

The present invention is directed to a novel method of performing enzymatic reactions involving magnesium dependent enzymes which are active at temperatures above 30°C, such as DNA polymerases, ligases, restriction endonucleases, and reverse transcriptases. Also provided is a novel method of performing hot start PCR reactions.
20 Furthermore, the present invention relates to achieving a greater specificity of these reactions. Also provided in the present invention are reagents and kits for performing these enzymatic reactions using a magnesium precipitate.

Background of the Invention

Polymerase Chain Reaction (PCR) is a rapid and simple method for specifically
25 amplifying a target DNA sequence in an exponential manner. Saiki, et al., *Science* 239:487-4391 (1988). Briefly, the method as now commonly practiced utilizes a pair of primers that have nucleotide sequences complementary to the DNA which flanks the target sequence. The primers are mixed with a solution containing the target DNA (the template), a thermostable DNA polymerase and deoxynucleoside triphosphates (dNTPS)
30 for all four deoxynucleotides (adenosine (A), tyrosine (T), cytosine (C) and guanine(G)).

5 The mix is then heated to a temperature sufficient to separate the two complementary strands of DNA. The mix is next cooled to a temperature sufficient to allow the primers to specifically anneal to sequences flanking the gene or sequence of interest. The temperature of the reaction mixture is then optionally reset to the optimum for the thermostable DNA polymerase to allow DNA synthesis (extension) to proceed. The temperature regimen is 10 then repeated to constitute each amplification cycle. Thus, PCR consists of multiple cycles of DNA melting, annealing and extension. Twenty replication cycles can yield up to a million-fold amplification of the target DNA sequence. In some applications a single primer sequence functions to prime at both ends of the target, but this only works efficiently if the primer is not too long in length. In some applications several pairs of 15 primers are employed in a process commonly known as multiplex PCR.

The ability to amplify a target DNA molecule by PCR has applications in various areas of technology e.g., environmental and food microbiology (Wernars et al., *Appl. Env. Microbiol.,* 57:1914-1919 (1991); Hill and Keasler, *Int. J. Food Microbiol.,* 12:67-75 (1991)), clinical microbiology (Wages et al. *J. Med. Virol.,* 33:58-63 (1991); Sacramento 20 et al., *Mol. Cell Probes,* 5:229-240 (1991)), oncology (Kumar and Barbacid, *Oncogene,* 3:647-651 (1988); McCormick, *Cancer Cells,* 1:56-61 (1989)), genetic disease prognosis (Handyside et al., *Nature,* 344:768-770 (1990)), and blood banking and forensics (Jackson, *Transfusion,* 30:51-57 (1990)).

Although significant progress has been made in PCR technology, the amplification 25 of non-target oligonucleotides due to side-reactions, such as mispriming on non-target background DNA, RNA, and/or the primers themselves, still presents a significant problem. This is especially true in diagnostic applications where PCR is carried out in a milieu containing complex background DNA and/or RNA while the target DNA may be

5 present at a very low level down to a single copy (Chou et al., *Nucleic Acid Res.,* 20:1717-1723 (1992)).

The temperature at which Taq DNA polymerase exhibits highest activity is in the range of 62° to 72° C., however, significant activity is also exhibited in the range of 20° to 37° C. As a result, during standard PCR preparation at ambient temperatures, the primers 10 may prime DNA extension at non-specific sequences because the formation of only a few base pairs at the 3'-end of a primer can result in a stable priming complex. The result can be competitive or inhibitory products at the expense of the desired product. As an example of inhibitory product, structures consisting only of primer, sometimes called "primer dimers" are formed by the action of DNA polymerase on primers paired with each other, 15 regardless of the true target template. The probability of undesirable primer-primer interactions increases with the number of primer pairs in the reaction, as with multiplex PCR. Other examples of inhibitory products are "wrong bands" of various length, caused by mispriming on the template DNA. During PCR cycling, these non-specific extension products can compete with the desired target DNA and/or lead to misinterpretation of the 20 assay.

Since these side reactions often occur during standard PCR preparation at ambient temperature, one method for minimizing these side reactions involves "hot start" PCR. Many PCR analyses, particularly the most demanding ones, benefit from a hot start. About 50% of all PCR reactions show improved yield and/or specificity if a hot start is 25 employed, and in some cases a hot start is absolutely critical. These demanding PCR analyses include those which have very low copy numbers of target (such as 1 HIV genome per 10,000 cells), denatured DNA (many DNA extraction procedures include a boiling step, so that the template is single- stranded during reaction setup), or contaminated DNA e.g., DNA from soil or feces and/or DNA containing large amounts of

5 RNA. However, current methods of achieving a hot start are tedious, expensive, and/or have other shortcomings.

Hot start PCR may be accomplished by various physical, chemical, or biochemical methods. In a physical hot start, the DNA polymerase or one or more reaction components that are essential for DNA polymerase activity is not allowed to contact the sample DNA

10 until all the components required for the reaction are at a high temperature. The temperature must be high enough so that not even partial hybridization of the primers can occur at any locations other than the desired template location, in spite of the entire genome of the cell being available for non-specific partial hybridization of the primers. Thus, the temperature must be high enough so that base pairing of the primers cannot

15 occur at template (or contaminating template) locations with less than perfect or near-perfect homology. This safe starting temperature is typically in the range of 50° to 75° C. and typically is about 10° C. hotter than the annealing temperature used in the PCR.

One physical way a hot start can be achieved is by using a wax barrier, such as the method disclosed in U.S. Pat. Nos. 5,599,660 and 5,411,876. See also Hebert et al., *Mol.*

20 *Cell Probes,* 7:249-252 (1993); Horton et al., *Biotechniques,* 16:42-43 (1994). Using such methods, the PCR reaction is set up in two layers separated by a 1 mm thick layer of paraffin wax which melts at about 56° C. There are several methods which may be used to separate the reaction components into two solutions. For instance, all of the DNA is added, with 1×buffer but no dNTPs and no DNA polymerase enzyme, in a volume of 25 ml. One

25 drop of melted wax is added and the tubes are all heated to 60° C. for one minute to allow the melted wax to form a sealing layer after which the tubes are cooled so the wax solidifies. Then a 25 ml mixture containing 1×buffer, all of the dNTPs, and the enzyme is added to each reaction. Finally, 1 drop of oil is added, to make 4 total layers. As the thermal cycler protocol heats the tubes to the first melting step (approximately 95° C.), the

5 wax melts and floats to mix with the oil layer, and the two aqueous layers mix by convection as the temperature cycles.

One common variation involving the use of a wax barrier is that the reaction components are assembled with no magnesium ions so that the DNA polymerase enzyme is inactive. The magnesium ion encased in a wax bead is then (or initially) added. A 10 further modification of the wax barrier used in PCR reactions is disclosed in the U.S. Pat. No. 5,599,660. Alternatively, at least one biological or chemical reagent needed for PCR is mixed with a wax carrier, resulting in a reagent that is solid at room temperature. Thus, the addition of other PCR reagents does not activate the DNA polymerase due to the fact that one or some of the reagents are sequestered in the wax. However, upon heating or the 15 addition of a solvent, the sequestered reagent(s) is/are released from the carrier wax and allowed to react with other soluble reagents, leading to the initiation of the PCR reaction. After the amplification is complete, the reactions are cooled to ambient temperature. Thus, a problem with these wax methods, however, is that the wax hardens after the completion of the amplification which makes sample recovery extremely tedious, since the wax tends 20 to plug the pipet tips used to remove the sample. This is true even if the samples are reheated to melt the wax. Another potential problem is cross-contamination if tweezers are used to add wax beads, since slight contact between the tweezers and the tube caps can move DNA template between samples before the PCR reactions start. Furthermore, the addition of a wax or a grease layer can negatively affect a PCR reaction since increasing 25 the total mass of the PCR reaction tube decreases the speed with which the contents of the tube approach the targeted temperatures in the thermal cycler.

Another way to implement a hot start PCR is to use DNA polymerase which is inactivated chemically but reversibly, such as AMPLITAQ GOLD® DNA polymerase. This enzyme preparation, distributed by PE Applied Biosystems, is distributed to users in

5 inactivated form, but is reactivatable by heating. The required reactivation conditions, however, are extremely harsh to the template DNA: ten minutes at 95° C. and at a nominal pH of 8.3 or lower results in reactivation of some 30% of the enzyme which is enough to start the PCR. See Moretti, et al., *Biotechniques* 25: 716-722 (1998). Because this treatment depurinates DNA every thousand bases or so, this enzyme can not be used to
10 amplify DNA more than a few kilobases in length. Accordingly, the use of this enzyme is most efficient when it is restricted to amplifying target DNA with a length of approximately 200 base pairs.

An additional way of implementing a hot start is to combine the Taq DNA polymerase enzyme with a Taq antibody before adding it to the reagent. This method
15 employs a monoclonal, inactivating antibody raised against Taq DNA polymerase. See Scalice et al., *J. Immun. Methods,* 172: 147-163 (1994); Sharkey et al., *Bio/Technology,* 12:506-509 (1994); Kellogg et al., *Biotechniques,* 16: 1134-1137 (1994). The antibody inhibits the polymerase activity at ambient temperature but is inactivated by heat denaturation. Unfortunately, the antibodies currently available for use in this method are
20 not very efficient, and a 5 to 10- fold molar excess must be used to effect the advantages of a hot start PCR. For Klentaq-278, an amino-terminally deleted *Thermus aquaticus* DNA polymerase that starts with codon 279 which must be used at higher protein levels for long PCR (up to ten times more protein than Taq DNA polymerase), the levels of antibody necessary for a hot start become extremely high and the denatured antibody protein retains
25 some inhibition for longer PCR targets. The original developer of anti-Taq antibodies (Kodak, now Johnson & Johnson) uses a triple-monoclonal antibody mixture which is more effective but is not commercially available and has not been tested in long PCR.

These methods used for hot starts require inclusion of an often expensive component (e.g., anti-Taq antibody) in the reaction mix and may place some undesirable

5 constraints on the performance of the PCR such as a relatively short time period between when a reagent is prepared and when it must be used, or a lower efficiency of amplification.

Yet another method used for hot start PCR is to specially design primers with secondary structures that prevent the primers from annealing until cycling temperatures 10 denature them. See Ailenberg et al., *Biotechniques,* 29: 1018-1020, 1022-1024 (2000). These specially designed primers are usually longer in length and special care must be taken in primer design. It may be inconvenient, expensive, or otherwise infeasible to design such primers.

Besides the grease/wax method, a low tech, inexpensive option of a physical hot 15 start is to add the enzyme, the magnesium and/or the dNTPs to the reactions after they have heated to a temperature sufficient to ensure specificity of primer annealing. This "manual" hot start method, besides being tedious and prone to error, commonly results in contamination and cross-contamination of PCR samples as the reaction tubes must be opened in the thermal cycler while they are hot.

20 Some PCR users believe they are performing a hot start when they set up PCR reactions in tubes on ice, then add the tubes to a thermal cycler block pre-warmed to 95° C. Although some benefit arises from this method, the addition of only a few nucleotides to a primer can take place every second during the fifteen seconds or more that the tubes warm from 0° to 25° C. This is enough to initiate unwanted competitive PCR for reactions 25 that require a hot start. Also, if many tubes are involved in an experiment, the tubes placed in the block first are heated for a longer time period at 95° C. compared to the tubes placed later in the heating block thus resulting in a lack of reproducibility between samples.

Therefore, the current methods of hot start PCR are associated with multiple shortcomings. In cases of applying physical methods of the hot start, the possible problems

5 include the ease of contamination, plugging up of pipet tips with wax or grease, and increase in time needed to reach target temperatures. In cases of applying chemical/biochemical methods of the hot start, the major drawbacks include the damage to template DNA resulting from harsh conditions needed to activate a chemically inactivated DNA polymerase, the excessive amounts of anti-Amplitaq antibody needed for
10 inactivation of a DNA polymerase prior to initiation of a PCR reaction, and significant costs of obtaining commercially available antibodies. Furthermore, the use of specially designed primers may place unnecessary constraints on PCR reactions.

Accordingly, a need exists for obtaining novel or modified methods of "hot start" PCR that would still provide all advantages of this procedure and at the same time
15 minimize or completely eliminate some of its shortcomings.

In addition to PCR technology, recombinant DNA technology generally has become widely used in recent years, has contributed to major scientific breakthroughs and relies heavily on the use of enzymes such as restriction endonucleases, ligases, and reverse transcriptases.

20 Restriction endonucleases naturally occur in bacteria, and isolated and purified forms of such nucleases can be used to “cut” DNA molecules at precise locations. These enzymes function by first recognizing and binding to a particular double-stranded sequence (“recognition sequence”) within the DNA molecule. Once bound, they cleave the DNA molecule either within or to one side of the recognition sequence to which they are
25 bound. The majority of restriction endonucleases recognize sequences that are four to six nucleotides in length; however, a small number of endonucleases can cleave sequences that are seven to eight nucleotides in length. The target DNA must be double-stranded for the restriction enzymes to bind and cleave. Apparent cleavage of single-stranded DNA is

5 actually due to the formation of double-stranded regions by intrastrand folding at ambient to warm temperatures (20° to 30°C).

The temperature at which restriction enzymes are active varies; however, many enzymes prefer temperatures above the ambient temperature. For example, 98% of enzymes available from New England BioLabs have optimum activities above 30°C. Some 10 5% of the restriction enzymes are active at temperatures above 55°C. All restriction endonucleases require magnesium ions for activity.

The second group of enzymes which are important in recombinant DNA technology are ligases. These enzymes are responsible for joining or ligating DNA molecules through a reaction involving the 3'-hydroxy and 5'-phosphate termini. *In vivo*, 15 one of the functions of DNA ligases involves fixing DNA damage which the ligase accomplishes by utilizing a molecule of ATP or NAD^+ to activate the 5' end at the nick in the DNA prior to forming a new bond. With regard to recombinant DNA molecules, the process is the same with the exception that the DNA ligase "seals" cohesive ends produced by restriction endonucleases instead of the nicks in the DNA. In case of blunt 20 ends, the ligation process is less efficient since base-pairing does not occur between the termini. Therefore, ligation reactions with blunt ends require higher concentrations of DNA and ligase in the reaction mixtures. *See* U.S. Patent No. 6,143,527.

In addition to ligation of recombinant DNA molecules, an important *in vitro* use of ligase is in ligase chain reaction (LCR) which is an alternative to PCR in target nucleic 25 acid amplification. LCR utilizes thermostable ligases, which are active at higher temperatures than regular ligases. For instance, Taq ligase, isolated from *Thermus aquaticus*, functions optimally at temperatures between 45°C and 65°C. In LCR reactions, repeated cycles of hybridization and ligation of primary and secondary probes result in amplification of the target sequence. *See* U.S. Patent No. 5,427,930. LCRs have been

5 utilized in DNA diagnostics such as genetic disease detection since they can detect single-base mismatches in DNA targets, thereby indicating the mutated or disease-causing alleles. *See* Barany, *Proc. Natl. Acad. Sci. USA*, Vol. 88, pp. 189-193, Jan. 1991.

One of the problems of achieving specificity in LCR is the ligation of the probe primers when they are non-specifically annealed to non-target DNA during reaction setup. 10 This can cause a seed of competing signal that confounds the specific detection and quantization of the desired specific sequence(s).

Reverse transcriptases (RT) were first recognized as components of retroviruses whose genetic material consists of single-stranded RNA. These viruses use RTs to synthesize a complementary DNA strand (cDNA) using viral RNA as a template, which is 15 followed by the synthesis of double stranded DNA and subsequent integration into the host genome. *See* U.S. Patent No. 5,998,195. At present, reverse transcriptases are frequently used in molecular biology because of their ability to synthesize complementary DNA from almost any RNA template. Thus, reverse transcriptase is commonly used to make nucleic acids for hybridization probes and to convert single-stranded RNA into a 20 single-stranded cDNA, which can further be converted into a double-stranded DNA for subsequent cloning and expression by techniques such as polymerase chain reaction (PCR).

Reverse transcriptases have been used as a component of transcription-based amplification systems that can amplify RNA and DNA target sequences up to 1 trillion 25 fold. *See e.g.*, PCT Patent Application WO 89/01050 and European Patent Application EP 0329822. Reverse transcriptases are also included in RT-PCR reactions wherein an initial step involves making a cDNA copy of the RNA target, which is then amplified by PCR. *See* U.S. Patent No. 5,998,195. Similarly to PCR reactions, RT-PCR reactions are very

5 sensitive to a variety of factors such as magnesium concentration and pH, and can result in production of nonspecific bands if RT can non-specifically initiate the synthesis of cDNA.

In addition, a method which would withhold a critical component, such as magnesium from magnesium dependent restriction endonucleases, ligases, and reverse transcriptases which are active above 30°C would be desirable to improve the specificity

10 of such enzymatic reactions. As such, a need exists to provide novel or modified methods of performing enzymatic reactions involving magnesium dependent enzymes that would allow for improved precision and specificity of the reactions.

Summary of the Invention

Among the several aspects of the invention, therefore, may be noted the provision

15 of novel processes for performing enzymatic reactions which require the use of a magnesium dependent enzyme such as DNA polymerase, a restriction enzyme, ligase or reverse transcriptase. These magnesium dependent enzymes are utilized in reactions which occur at temperatures above 30°C. Briefly, the present invention is directed to processes of synthesizing nucleic acids using DNA polymerases, cleaving DNA using restriction

20 endonucleases, ligating DNA using DNA ligases, and transcribing RNA into cDNA using reverse transcriptases. Accordingly, the present invention provides reagents and kits which can be used to perform said reactions.

In particular, the processes of the invention comprise sequestering magnesium ions in a precipitate thereby rendering the magnesium dependent enzyme such as a restriction

25 endonuclease, a ligase, or a reverse transcriptase inactive until the magnesium ions are released. In one aspect, the processes of the present invention utilize a reagent which comprises a precipitate containing magnesium. Alternatively, the reagent comprises a source of magnesium ions with a source of phosphate ions which can be used to form a precipitate combining the source of magnesium ions and the source of phosphate ions at a

5 temperature of below 34°C. These reagents are utilized in enzymatic reactions including cleaving of DNA, reverse transcribing DNA and ligating DNA molecules, which occur at temperatures above 30°C in order to improve the specificity of such reactions.

A further aspect of the present invention is to provide kits useful for reactions involving magnesium dependent enzymes. These enzymes include restriction 10 endonucleases, ligases and reverse transcriptases. In one embodiment, kits of the present invention comprise a container containing a source of magnesium ions and a container containing a source of phosphate ions which form a precipitate containing magnesium when combined at temperatures of below 34°C and instructions for performing said reactions. In another embodiment, the kits comprise a container containing a reagent 15 comprising a precipitate containing magnesium and instructions for using the precipitate containing magnesium. Preferably, other reagents necessary for the above-mentioned reactions are included in the kits of the present invention.

Among the several aspects of the invention, therefore, may be noted the provision of novel processes for performing hot start PCR reactions. Briefly, the present invention is 20 directed to processes for synthesizing nucleic acid extension products and specifically, to methods for amplifying a target nucleic acid sequence using PCR. Accordingly, the present invention provides reagents and kits which can be used to synthesize a nucleic acid extension product.

As such, it is an aspect of the present invention to increase the specificity of PCR 25 product amplification by providing a new method for hot start PCR. In particular, the processes comprise sequestering magnesium ions in a precipitate thereby rendering the DNA polymerase inactive until the magnesium ions are released. In one aspect, the processes of the present invention utilize a reagent which comprises a precipitate containing magnesium. Alternatively, the precipitate is formed by combining a source of

5 magnesium ions and a source of phosphate ions at a temperature of 4° to 30° C. The precipitate is combined with the PCR reaction components e.g., a thermostable DNA polymerase, deoxyribonucleoside triphosphates, a set of primers and a target nucleic acid sequence. The magnesium ions are then released from the precipitate, preferably by heating the mixture to a temperature sufficient to release the magnesium ions from the
10 precipitate and into the mixture. The release of magnesium ions into the mixture activates the DNA polymerase thus allowing the extension of each primer to proceed.

A further aspect of the present invention is to provide kits for amplifying a target nucleic acid. In one embodiment, kits of the present invention comprise a container containing a source of magnesium ions and a container containing a source of phosphate
15 ions which form a precipitate containing magnesium when combined at a temperature of 4° to 30° C., and instructions for amplifying the target nucleic acid. In another embodiment, the kits comprise a container containing a reagent comprising a precipitate containing magnesium and instructions for using the precipitate containing magnesium to amplify the target nucleic acid sequence. Preferably, other reaction reagents such as a
20 DNA polymerase or a mixture of DNA polymerases and deoxyribonucleoside triphosphates are included in the kits of the present invention.

Other aspects and features will be in part apparent and in part pointed out hereinafter.

Brief Description of the Figures

25 These and other features, aspects, and advantages of the present invention will become better understood with regard to the following description, claims and accompanying drawings where:

FIG. 1 is an image of an agarose gel depicting the amplification products of hot start PCR reactions performed using different concentrations of phosphoric acid (3-7 mM)

5 added to magnesium chloride in TAT buffer. Lanes 1 and 2 represent the standard molecular weight ladders. The PCR reactions in lanes 3 and 6 were performed by a manual hot start method and the PCR reactions in the remaining lanes were prepared at room temperature and incubated at 30° C. for 30 minutes (warm start). The PCR products represented in lanes 3-5 were formed without any phosphoric acid in the TAT buffer

10 whereas the products in other lanes (6-16) were formed utilizing TAT buffer containing phosphoric acid ranging in concentration from 3 mM to 7 mM (TAT3-TAT7). In lanes 7-16, this buffer was initially incubated with magnesium chloride to form the precipitate. The superscript represents the milimolar concentrations of phosphoric acid in the PCR reactions.

15 FIG. 2 is an image of an agarose gel depicting the amplification products of hot start PCR reactions performed using different concentrations of phosphoric acid (5-19 mM) added to magnesium chloride in TAT buffer. Lanes 1 and 2 represent the standard molecular weight markers. Lanes 3, 4, 7, and 8 are representations of a manual hot start, whereas the standard/bench start is portrayed in lanes 5 and 6. The remaining lanes contain

20 the products from PCR reactions that were performed by using the magnesium precipitate hot start method. Specifically, lanes 9-19 depict PCR products that were formed as a result of PCR reactions utilizing TAT buffers containing different phosphoric acid concentrations. The superscript represents the milimolar concentrations of phosphoric acid in each PCR reaction.

25 FIG. 3 is an image of an agarose gel of a magnesium precipitate hot start PCR using TaqLA and KlentaqLA. Lanes 1 and 2 represent the standard molecular weight markers. Lanes 3 and 4 and lanes 9 and 10 represent the products of the manual hot start method performed with KlentaqLA and TaqLA, respectively. Lanes 5 and 6 and lanes 11 and 12 show minimal amplification of the products when using regular PCR methods

5 (bench start) using KlentaqLA and TaqLA, respectively. Lanes 7 and 8 and lanes 13 and 14 illustrate the use of KlentaqLA and TaqLA in magnesium precipitate hot start reactions. The superscript represents the milimolar concentrations of phosphoric acid in the PCR reactions.

FIGS. 4A and 4B are images of agarose gels depicting the magnesium precipitate

10 hot start method utilizing different magnesium comprising compounds including magnesium chloride ($MgCl_2$), magnesium sulfate ($MgSO_4$), magnesium hydroxide ($Mg(OH)_2$) and magnesium carbonate ($MgCO_3$). In FIG. 4A , lanes 1 and 2 represent the standard molecular weight markers. The PCR reactions from lanes 3-8 were performed in the presence of magnesium chloride, lanes 9-14 were performed in the presence of

15 magnesium sulfate, and lanes 15-20 were done in the presence of magnesium hydroxide. In FIG. 4B , lanes 1 and 2 represent the standard markers and lanes 3-6 were performed in the presence of magnesium carbonate. A manual hot start is represented by lanes 3, 4, 9, 10, 15 and 16 in FIG. **4** A and lanes 3 and 4 in FIG. **4** B. The regular bench start is shown in lanes 5, 6, 11, 12, 17 and 18 of FIG. **4** A and lane 5 in FIG. **4** B. The magnesium

20 precipitate hot start is depicted in FIG. 4A in lanes 7, 8, 13, 14, 19, and 20, and lane 6 in FIG. **4** B. The superscript represents the milimolar concentrations of the phosphoric acid in the PCR reactions.

FIGS. 5A and 5B are images of agarose gels depicting the magnesium precipitate hot start method utilizing different phosphate containing compounds, including H_3PO_4,

25 KH_2PO_4, NaH_2PO_4, and $CH_6O_6P_2$. In FIG. 5A , lanes 1 and 2 are the molecular weight markers. The PCR reactions in lanes 3-6 were performed using TAT buffer (no phosphate), whereas the PCR reactions depicted in lanes 7-10 were performed using TAT buffer containing 5 mM phosphoric acid. Furthermore, lanes 3, 4, 7, and 8 were performed as manual hot start reactions, whereas lanes 5, 6, 9 and 10 were performed with the

5 magnesium precipitate hot start method. In FIG. 5B , lanes 1 and 2 are the molecular weight markers and lanes 3-6 depict PCR reactions performed using 5 mM KH 2 PO 4 . Lanes 7-10 represent the PCR reactions that were performed using 5 mM NaH 2 PO 4 and the PCR reactions depicted in lanes 11-14 were performed using 5 mM methylenediphosphonic acid (MDP). In FIG. 5B , lanes 3, 4, 7, 8, 11, and 12 represent 10 manual hot start reactions, and lanes 5, 6, 9, 10, 13, and 14 represent magnesium precipitate hot start PCRs.

FIG. 6 is an image of an agarose gel depicting the effect of magnesium chloride concentration on precipitate formation and the efficacy of the magnesium precipitate hot start PCR. Lane 1 is a standard molecular weight marker, lanes 2-11 were performed as 15 manual hot start PCRs in the presence of the TAT buffer and increasing concentrations of magnesium chloride and lanes 12-21 were performed as magnesium precipitate hot start reactions in the presence of TAT5 buffer and increasing magnesium chloride concentrations. The superscript represents the final milimolar concentrations of the magnesium chloride used in the PCR reactions.

20 FIG. 7 is an image of an agarose gel depicting the effect of incubating TAT5 buffer with magnesium chloride for various lengths of time. Lanes 1 and 2 represent molecular weight markers, lanes 3 and 4 depict the manual hot start method and lanes 5 and 6 represent the regular bench start. Lanes 7-16 depict the products of the magnesium precipitate hot start PCR reactions. TAT5 and magnesium chloride were allowed to 25 incubate during the magnesium precipitate hot start reactions for 15 minutes (lanes 7 and 8), 10 minutes (lanes 9 and 10), 5 minutes (lanes 11 and 12), 2 minutes (lanes 13 and 14), and 0 minutes (lanes 15 and 16). The superscripts represent minutes of incubation of TAT5 and magnesium chloride.

5 FIG. 8 is an image of an agarose gel depicting use of the magnesium precipitate hot start method to amplify HIV-1 gag gene. Lanes 1 and 2 depict the molecular markers, lanes 3 and 4 depict the manual hot start, lanes 5 and 6 depict the bench start method, and lanes 7 and 8 depict the magnesium precipitate hot start reaction. The bench start method of performing PCR resulted in the amplification of an incorrect band thus showing the

10 lack of specificity. However, both the manual and magnesium precipitate hot starts yielded the bands of correct size. The superscript represents the milimolar concentration of phosphoric acid utilized in the reaction.

FIG. 9 is an image of an agarose gel showing the effect of ammonium sulfate ((NH 4) 2 SO 4) in the TAT buffer on the formation of precipitate and subsequent PCR product

15 (HIV-1 gag) amplification. Lanes 1 and 2 represent the standard molecular weight markers, lanes 3 and 4 represent the manual hot start reactions, and the lanes 5 and 6 represent the bench start. The bench start yielded the wrong band thus indicating the advantage of applying the hot start methods in PCR reactions. Lanes 7- 10 represent the magnesium precipitate hot start PCR reactions. The incubation of the phosphoric acid with

20 magnesium chloride was done so that either ammonium sulfate or both Tris and ammonium sulfate were excluded from the buffer. The missing reagents were then added to the reaction tubes with mastermixes prior to running the reactions. Lanes 7 and 8 represent withholding both Tris and ammonium sulfate during the incubation step whereas in the PCR reactions depicted in lanes 9 and 10, only ammonium sulfate was excluded

25 during the incubation.

FIG. 10 is an image of an agarose gel depicting the optimal concentration of ammonium phosphate ((NH 4) 2 HPO 4) for use in the magnesium precipitate hot start PCR reactions. All lanes show the amplification of *Cryptosporidium parvum* heat shock protein homolog gene (hsp70). Lanes 1 and 2 represent the standard molecular markers.

5 Lanes 3 and 4 and lanes 5 and 6 represent the manual hot start and the bench start, respectively. As shown in lanes 7 and 8, the manual hot start was also performed with TAT buffer containing ammonium phosphate as the source of phosphate ions. The remaining lanes (9-18) depict the amplification products in magnesium precipitate hot start reactions that utilized ammonium phosphate. In these reactions, only the concentration of
10 phosphate ions was increased while the concentration of ammonium ions was kept constant. The concentrations of phosphate that were tested are represented by superscripts and include: 1 mM (lanes 9 and 10), 3 mM (lanes 11 and 12), 5 mM (lanes 13 and 14), 7 mM (lanes 15 and 16), and 10 mM (lanes 17 and 18).

Detailed Description

15 All publications, patents, patent applications or other references cited in this application are herein incorporated by reference in their entirety as if each individual publication, patent, patent application or reference are specifically and individually indicated to be incorporated by reference.

Abbreviations and Definitions

20 The listed abbreviations and terms, as used herein, are defined as follows:

bp is the abbreviation for base pairs.

kb is the abbreviation for kilobase (1000 base pairs).

nt is the abbreviation for nucleotides.

Taq is the abbreviation for *Thermus aquaticus.*

25 Pfu is the abbreviation for *Purococcus furiosus.*

Tth is the abbreviation for *Thermus thermophilus.*

"Stoffel fragment" refers to a DNA polymerase having substantially the same amino acid sequence as *Thermus aquaticus* DNA polymerase but lacks the 5' nuclease

5 activity due to a genetic manipulation which results in the deletion of the N-terminal 289 amino acids of the polymerase molecule. See Erlich et al., Science 252:1643, 1991.

"Deep Vent" DNA polymerase is purified from an archael, thermophilic bacterium by New England Biolabs, Inc.

"Klentaq1" is a trademarked commercial name for Klentaq-278 which is a DNA
10 polymerase having substantially the same amino acid sequence as *Thermus aquaticus* DNA polymerase, but excluding the N-terminal 278 amino acids, ±one residue as claimed in U.S. Pat. No. 5,616,494, incorporated herein by reference.

"LA PCR" is Long and Accurate PCR using an unbalanced mixture of two DNA polymerases, as claimed in U.S. Pat. No. 5,436,149.

15 "KlentaqLA" is an unbalanced mixture of two DNA polymerases, wherein the major component is the thermostable DNA polymerase known as Klentaql or Klentaq278 and lacking 3'-exonuclease activity and the minor component is at least one DNA polymerase exhibiting 3'-exonuclease activity, as claimed in U.S. Pat. No. 5,436,149. KlentaqLA is commercially available from Clontech (Cat. No. 8421-1) and from Sigma
20 (Cat. No. D6290). In the examples shown, the minor component is "Deep Vent" DNA polymerase.

"TaqLA" is an unbalanced mixture of two DNA polymerases, wherein the major component is full-length Taq DNA polymerase as the thermostable DNA polymerase lacking 3'-exonuclease activity and the minor component is at least one DNA polymerase
25 exhibiting 3'-exonuclease activity, as claimed in U.S. Pat. No. 5,436,149, incorporated herein by reference. In the examples shown, the minor component is "Deep Vent" DNA polymerase.

5 "Thermostable" is defined herein as having the ability to withstand temperatures up to at least 95° C. for many minutes without becoming irreversibly denatured and the ability to polymerize DNA at optimum temperatures of 55° C. to 75° C.

In vitro processes of producing replicate copies of the same polynucleotide, such as PCR, are collectively referred to herein as "amplification" or "replication." For example, 10 single or double stranded DNA may be replicated to form another DNA with the same sequence. RNA may be replicated, for example, by a RNA directed RNA polymerase, or by reverse transcribing the RNA using a reverse transcriptase or a DNA polymerase exhibiting reverse transcriptase activity and then performing a PCR amplification. In the latter case, the amplified copy of the RNA is a DNA (known as "complementary DNA" or 15 "cDNA") with the correlating or homologous sequence.

The polymerase chain reaction ("PCR") is a reaction in which replicate copies are made of a target polynucleotide using one or more primers, and a catalyst of polymerization, such as a DNA polymerase, and particularly a thermally stable polymerase enzyme. Generally, PCR involves repeatedly performing a "cycle" of three 20 steps: "melting", in which the temperature is adjusted such that the DNA dissociates to single strands, "annealing", in which the temperature is adjusted such that oligonucleotide primers are permitted to match their complementary base sequence using base pair recognition to form a duplex at one end of the span of polynucleotide to be amplified; and "extension" or "synthesis", which may occur at the same temperature as annealing, or in 25 which the temperature is adjusted to a slightly higher and more optimum temperature, such that oligonucleotides that have formed a duplex are elongated with a DNA polymerase. This cycle is then repeated until the desired amount of amplified polynucleotide is obtained. Methods for PCR amplification are taught in U.S. Pat. Nos. 4, 683,195 and 4,683,202.

5 "Specificity" in PCR amplification refers to the generation of a single, "specific," PCR product with the size and sequence predicted from the sequences of the primers and the genomic or transcribed region of nucleic acid to which the primers were designed to anneal in a base- complementary manner. "Nonspecific" PCR product has a size or sequence different from such prediction. A "target nucleic acid" is that genomic or 10 transcribed region of nucleic acid, the ends of which are base- complementary (with proper orientation) to primers included in a complete set of PCR reagents. A primer refers to a nucleic acid sequence, which is complementary to a known portion of a target nucleic acid sequence and which is necessary to initiate synthesis by DNA polymerase. "Proper orientation" is for the two primers to anneal to opposite strands of double-stranded target 15 nucleic acid with their 3' ends pointing toward one another. Such primers are said to target the genomic or transcribed sequence to the ends of which they are base-complementary. An "appropriate temperature", as referred to in the claims in regard to the PCR amplifications, indicates the temperature at which specific annealing between primers and a target nucleic acid sequence occurs.

20 "Manual hot start PCR" is a PCR method that generally produces improved reliability, improved products from low-copy targets, and/or cleaner PCR products. Template DNA and primers are mixed together and held at a temperature above the threshold of non-specific binding of primer to template. All of the PCR reaction components are added to the extension reaction except one critical reagent which is 25 withheld. The withheld reagent is usually the thermostable polymerase or the magnesium, but it can also be, for instance, the triphosphates or the primers. Just prior to the cycling, the withheld reagent is added to allow the reaction to take place at higher temperature. Due to lack of non-specific hybridization of primers to template or to each other, the PCR

5 amplification proceeds more efficiently as a result of the reduction or elimination of competing extensions at non-target locations.

"Standard or bench start" are used interchangeably herein and when used to refer to PCR amplification, indicate that all the PCR reaction components needed for amplification are added to the template nucleic acid sequence at 25° C.

10 "Warm start" is used herein and when used to refer to PCR amplification, indicates that all the PCR reaction components needed for amplification are added to the template nucleic acid sequence at 25° C. followed by an incubation at 30° for 30 minutes.

When referring to a particular DNA polymerase, the term "polymerase activity" refers to the ability of the DNA polymerase to incorporate dNTPs or ddNTPS in a chain

15 extension reaction.

“Reverse transcription”, “reverse transcribing” or “RT reaction” refers to the process by which RNA is converted into cDNA through the action of a nucleic acid polymerase such as reverse transcriptase. Methods for reverse transcription are well known in the art and described for example in Fredrick M. Ausubel et al. (1995), "Short

20 Protocols in Molecular Biology," John Wiley and Sons, and Michael A. Innis et al. (1990), "PCR Protocols," Academic Press.

"*Thermus aquaticus* DNA polymerase" or "Taq DNA polymerase" are used interchangeably to refer to heat stable DNA polymerases from the bacterium *Thermus aquaticus* and include all Taq mutants, natural and synthesized.

25 “Reverse transcriptase” is defined herein as an RNA-directed DNA polymerase or as a DNA polymerase exhibiting reverse transcriptase ability.

rTth is the abbreviation for recombinant thermostable polymerase obtained from *Thermus thermophilus* that possesses reverse transcriptase and Taq-like DNA polymerase activities.

5 "RT-PCR" or "reverse transcriptase polymerase chain reaction" is a reaction in which replicate DNA copies are made of a target RNA sequence using one or more primers, and catalysts of polymerization, such as reverse transcriptase and DNA polymerase, and particularly thermostable forms of these enzymes. Generally, a target RNA sequence is first reverse transcribed into cDNA by the action of reverse 10 transcriptase. Subsequently, PCR is performed, wherein the cDNA can be amplified many times depending on the number of PCR cycles. For instance, twenty amplification cycles can yielded up to a million-fold amplification of the target DNA sequence. Methods for PCR amplification are taught in U.S. Pat. Nos. 4,683,195 and 4,683,202. For RT-PCR, *see e.g.*, U.S. Patent Nos. 5,130,238 and 5,693,517.

15 "Single restriction enzyme digest" or "restriction enzyme reaction" are used interchangeably herein to refer to reactions catalyzed by a single restriction enzyme that cleaves target DNA at specific sites either within or at the ends of DNA molecule(s).

"Multiple restriction enzyme digest" or "multiple restriction enzyme reaction" are used interchangeably herein to indicate reactions catalyzed by multiple restriction 20 enzymes that cleave target DNA molecule at their cognate sites either within or at the ends of the DNA molecules.

"Ligase reaction" as used herein refers to a reaction catalyzed by a ligase, which results in ligation or joining of target nucleic acid sequences through formation of phosphodiester bonds between 5' and 3' termini of the target nucleic acids.

25 "Specificity" in RT-PCR reaction refers to the generation of a single, "specific", RT-PCR product with the size and sequence predicted from the sequences of the primers and the genomic or transcribed region of nucleic acid to which the primers were designed to anneal in a base-complementary manner. "Specificity" in a single or a multiple restriction enzyme digest refers to the ability of restriction enzyme(s) to only cleave DNA

5 at their cognate recognition sequences in double-stranded form without cleaving any other similar, non-specific or single-stranded DNA sequences. "Specificity" in a ligase reaction refers to the ability of the ligase to specifically join two or more DNA sequences only when their 5' and 3' ends being joined are fully double-stranded and base-paired for at least few bases or for the length of the oligonucleotide substrate probes.

10 The present invention further provides processes and kits for performing reactions requiring magnesium dependent enzymes. Preferably, these enzymes comprise ligases, restriction endonucleases, and reverse transcriptases. The enzymes utilizes in these processes are magnesium dependent and the enzymatic reactions in which the enzymes are utilized occur at temperatures above 30°C. The processes and kits utilize the step of 15 sequestering magnesium ions, thereby rendering a magnesium dependent enzyme inactive until the magnesium ions are released from the precipitate into the reaction mixture.

The magnesium precipitate method of the present invention is achieved by forming a precipitate comprising magnesium ions which sequesters the magnesium ions from other reaction reagents and preferably, prevents significant magnesium dependent enzyme 20 activity due to the lack of magnesium ions in the reaction mixture. The magnesium ions utilized in the present invention are available from different sources. Preferably, the sources of magnesium ions include but are not limited to magnesium chloride, magnesium hydroxide, magnesium carbonate and magnesium sulfate. In a preferred embodiment, the source of magnesium ions is magnesium chloride.

25 Many sources of phosphate ions are available in the art. Preferably, the sources of phosphate ions include but are not limited to phosphoric acid (H_3PO_4), potassium phosphate (K_2HPO_4), and ammonium phosphate ($(NH_4)_2HPO_4$). In a preferred embodiment, the source of phosphate ions is ammonium phosphate or phosphoric acid and more preferably, the source of phosphate ions utilized is phosphoric acid.

5 Many buffers used in reactions utilizing restriction enzymes, ligases, or reverse transcriptases contain magnesium. As such, the processes of the present invention may utilize buffers which contain the source of magnesium ions for the formation of the magnesium precipitate. In this embodiment, the magnesium precipitate method is achieved by adding a source of phosphate ions to a buffer containing magnesium ions to form a 10 precipitate containing magnesium. Preferably, this buffer containing magnesium ions is at higher concentration *i.e.*, contains less water, than the concentration of the reaction mixture at which the enzymatic process occurs.

In a preferred embodiment, the source of phosphate ions is contained in a solution which is buffered to a pH above 7. Solutions or buffers used for performing reactions with 15 magnesium dependent enzymes vary depending on the enzyme used. For ligase reactions, the buffer often comprises Tris (for pH stabilization), a source of magnesium ions, a reducing agent, preferably dithiothreitol (DTT), and bovine serum albumin (BSA) or a surfactant for preventing aggregation of enzyme, a salt, preferably potassium acetate. If Taq ligase the ligase utilized in the reaction, then the buffer will also contain NAD+ co-20 factor. For RT-PCR reactions, the buffer commonly comprises Tris, a source of magnesium ions, a reducing agent such as DTT, and a salt such as potassium chloride. Buffers for restriction enzymes vary in specific content but commonly contain Tris, a salt, usually sodium chloride or potassium acetate, and a reducing agent such as DTT. The required concentrations of these buffer components will vary depending on the magnesium 25 dependent enzyme. Such concentrations would be easily determined by one skilled in the art.

Alternatively, buffers may be utilized in the enzymatic process which are not pre-formulated with a source of magnesium or a source of phosphate ions. In this case, either the source of magnesium ions or the source of phosphate ions can first be mixed with the

5 buffer and incubated with either the source of phosphate ions or the source of magnesium ions, respectively, to form a precipitate containing magnesium. This is another way of achieving all the benefits of magnesium precipitate method for magnesium dependent enzymes.

The precipitate is formed by combining a source of magnesium ions and a source
10 of phosphate ions for at least 3 minutes at a temperature below 34°C, preferably ranging from 4° to 30°C and preferably, at 4°C. The incubation of phosphoric acid with magnesium ions for approximately 3 minutes at a low temperature produces an insoluble precipitate containing magnesium and phosphate. Preferably, the source of magnesium ions and the source of phosphate ions are incubated at a temperature of at least 4°C. In
15 another preferred embodiment, the source of magnesium ions and the source of phosphate ions are incubated at a temperature of at least 25°C. In yet another preferred embodiment, the source of magnesium ions and the source of phosphate ions are incubated at a temperature of 0° to 30°C. The source of magnesium and the source of phosphate are incubated for at least three minutes to form the precipitate containing magnesium.
20 Preferably, the source of magnesium and the source of phosphate are incubated for at least 5 minutes and more preferably, for at least 10 minutes.

In a preferred embodiment, the source of phosphate ions is incubated with a source of magnesium ions in a concentration at or above appropriate for a particular enzyme and for a particular enzymatic reaction, at a temperature of 4° to 30°C for at least 5 minutes,
25 more preferably 15 minutes, to form a precipitate containing magnesium.

Once the precipitate is formed, the additional reagents appropriate for the enzymatic reaction being performed are added. In case of single or multiple restriction enzyme digests, the commonly added reagents include sterile nuclease-free water, a target DNA sample, and restriction enzyme(s). For ligase reactions, the additional reagents to be

5 added are target DNA molecule(s), and a particular ligase, preferably Taq ligase. If Taq ligase is utilized, then co-factor NAD+ is also added to the reaction mixture. RT reactions would require addition of a target RNA sequence, at least one primer, deoxyribonucleosides, and a reverse transcriptase. Hot start RT-PCR reactions require the addition of a target RNA sequence, at least one primer, deoxyribonucleosides, and an
10 enzyme or mixture of enzymes possessing both RT and DNA polymerase activities (such as rTth) .

After the precipitate is combined with other reaction reagents to form a reaction mixture, the magnesium is released from the precipitate and into the reaction mixture. The release of the magnesium ions into the reaction mixtures results in making the magnesium
15 available to the enzyme and consequentially, activating the magnesium dependent enzyme for the desired enzymatic process. The ability of the precipitate to sequester magnesium until the appropriate conditions are achieved to release the magnesium results in increased specificity of the reaction and/or simultaneous start of a number of reactions. Preferably, the mixture containing the precipitate and reaction reagents is heated to standard
20 temperatures required for the reaction being performed so that the magnesium is released from the precipitate at a higher temperature than the temperature at which nonspecific DNA ligation, digestion or RNA reverse transcription occur, and more preferably, the magnesium ions are released by heating the reaction mixture to a temperature above 30°C. In this way, the magnesium precipitate method provides an improved specificity for
25 reactions involving magnesium dependent enzymes. The temperature at which the precipitate dissolves is achieved during the standard reaction temperatures, thereby eliminating any extra steps and need for additional reagents.

Besides a greater precision and specificity, the magnesium precipitate method possesses other beneficial attributes such as the ease of manipulation, the little extra time

5 necessary to perform it, and the inexpensive reagents required. The processes of the present invention are not only useful in reactions specified above, but can also be applied in any reaction that requires use of a magnesium dependent enzyme.

A further aspect of the present invention is to provide kits useful for reactions involving magnesium dependent enzymes. These enzymes include restriction
10 endonucleases, ligases and reverse transcriptases. In one embodiment, kits of the present invention comprise a container containing a source of magnesium ions and a container containing a source of phosphate ions which form a precipitate containing magnesium when combined at temperatures of below 34°C and instructions for performing said reactions. In another embodiment, the kits comprise a container containing a reagent
15 comprising a precipitate containing magnesium and instructions for using the precipitate containing magnesium. Preferably, other reagents necessary for the above-mentioned reactions are included in the kits of the present invention.

The procedures disclosed herein which involve the molecular manipulation of nucleic acids are known to those skilled in the art. See generally Fredrick M. Ausubel et
20 al. (1995), "Short Protocols in Molecular Biology," John Wiley and Sons, and Joseph Sambrook et al. (1989) , "Molecular Cloning, A Laboratory Manual," second ed., Cold Spring Harbor Laboratory Press, which are both incorporated by reference.

Accordingly, the present invention provides processes and kits for performing a "hot start" PCR. The processes and kits utilize the step of sequestering magnesium ions in
25 a precipitate prior to the extension step of the PCR reaction thereby rendering a DNA polymerase inactive until the mixture the magnesium ions are released from the precipitate. As a result, amplification of target DNA molecules is specific with minimal or no formation of competitive or inhibitory products. Thus, the processes and kits for amplification of a nucleic acid have improved efficacy which is achieved by preventing a

5 significant catalytic reaction of DNA polymerase with other reagents until the extension cycle of PCR.

The hot start PCR of the present invention is achieved by forming a precipitate comprising magnesium ions which sequesters the magnesium ions from other PCR reagents and preferably, prevents significant DNA polymerase activity due to the lack of

10 magnesium ions in the reaction mixture. As such, the magnesium ions required for DNA polymerase activity are withheld from the DNA polymerase and other PCR reagents prior to the transfer of the tubes into the thermal cycler. The precipitate is formed by combining a source of magnesium ions and a source of phosphate ions for at least 3 minutes at a temperature ranging from 4° to 30° C. The magnesium ions utilized in the present

15 invention are available from different sources. Preferably, the sources of magnesium ions include but are not limited to magnesium chloride, magnesium hydroxide, magnesium carbonate and magnesium sulfate. In a preferred embodiment, the source of magnesium ions is magnesium chloride, which is most commonly used in PCR reactions. The concentration of magnesium needed for the magnesium precipitate hot start is similar to

20 the concentration that is needed for a manual hot start. Preferably, the concentration of magnesium chloride in the present invention is about 3.5 mM.

Many sources of phosphate ions are available in the art. Preferably, the sources of phosphate ions include but are not limited to phosphoric acid (H_3PO_4), potassium phosphate (K_2HPO_4), and ammonium phosphate ($(NH_4)_2HPO_4$). In a preferred

25 embodiment, the source of phosphate ions is ammonium phosphate or phosphoric acid and more preferably, the source of phosphate ions utilized is phosphoric acid. The concentrations of the phosphoric acid that are suitable for magnesium precipitate hot start range from about 3 mM to 13 mM and preferably, the concentration of phosphoric acid is

5 between 5 mM and 7 mM. Alternatively, if ammonium phosphate is the source of the phosphate ions, the concentration of ammonium phosphate ranges from 2 mM to 6 mM.

Preferably, the source of phosphate ions is contained in a solution which is buffered to a pH above 7. In a preferred embodiment, the buffer contains Tris (Tris(hydroxymethyl)aminomethane) and more preferably, ammonium sulfate to aid in the
10 production of the precipitate during the magnesium precipitate hot start method. Preferably, the buffer used in the present invention is TAT buffer (50 mM Tris-HCl with pH 9.2, 16 mM ammonium sulfate, and 0.1% Tween 20) having a final pH of approximately 9. 1. In a preferred embodiment, the source of the phosphate ions is premixed with the TAT buffer prior to the formation of the precipitate. Accordingly, when
15 TAT buffer containing a source of phosphate ions, ammonium sulfate, and Tris is incubated with a source of magnesium ions, ammonium sulfate and Tris contribute to formation of the precipitate. Preferably, the TAT buffer used in the processes employs phosphoric acid as the source of phosphate ions. As shown in the Example 8, ammonium sulfate and Tris are not essential for the formation of the precipitate and execution of the
20 magnesium precipitate hot start; however, the use of ammonium sulfate and Tris in the buffer enhances the precipitation reaction and the amplification of the products.

The incubation of phosphoric acid with magnesium ions in the presence of TAT buffer for approximately 3 minutes at a low temperature, preferably below 34° C., produces an insoluble precipitate comprising magnesium and phosphate. In yet another
25 preferred embodiment, the source of magnesium ions and the source of phosphate ions are incubated at a temperature of 0° to 30° C. Preferably, the source of magnesium ions and the source of phosphate ions are incubated at a temperature of at least 4° C. In another preferred embodiment, the source of magnesium ions and the source of phosphate ions are incubated at a temperature of at least 25° C. The source of magnesium and the source of

5 phosphate are incubated for at least three minutes to form the precipitate containing magnesium. Preferably, the source of magnesium and the source of phosphate are incubated for at least 5 minutes and more preferably, for at least 10 minutes.

Once the precipitate is formed, the additional PCR reaction components are added. Such PCR reagents include at least one DNA polymerase, deoxyribonucleoside
10 triphosphates, at least one primer and at least one target nucleic acid sequence. Preferably, the DNA polymerases are thermally stable DNA polymerases. Some examples of thermally stable DNA polymerases include, but are not limited to, *Thermus aquaticus* DNA polymerase, N-terminal deletions of Taq DNA polymerase such as Stoffel fragment DNA polymerase, Klentaq235, and Klentaq-278 ; *Thermus thermophilus* DNA
15 polymerase; *Bacillus caldotenax* DNA polymerase; *Thermus flavus* DNA polymerase; *Bacillus stearothermophilus* DNA polymerase; and archaebacterial DNA polymerases such as *Thermococcus litoralis* DNA polymerase (also referred to as Vent), Pfu, Pfx, Pwo, and Deep Vent or a mixture of DNA polymerases. In a preferred embodiment, the DNA polymerases are TaqLA, KlentaqLA, Klentaq1, Pfu, Deep Vent or Tth. More preferably,
20 the DNA polymerases are TaqLA, Klentaq1, and KlentaqLA.

After the precipitate is combined with other PCR reagents, the magnesium ions are released from the precipitate thereby activating the DNA polymerase. Preferably, the magnesium ions are released from the precipitate by heating the reaction mixture to a temperature above 30° C. However, other methods may be used to release the magnesium
25 ions from the precipitate and into the reaction mixture e.g., chemical reactions, pH changes. The ability to quickly release magnesium ions from the precipitate results in the amplification of the specific target nucleic acid sequence with minimal or no formation of competitive or inhibitory products. As DNA polymerases require magnesium in order to synthesize nucleic acid extension products, the release of the magnesium ions into the

5 mixture results in the extension of the target nucleic acid molecules. Preferably, the mixture containing the precipitate and PCR reaction reagents is heated to standard cycling temperatures (50°-95° C., preferably 68° C.) so that the magnesium is released from the precipitate at a higher temperature than the temperature at which mispriming occurs. In this way, the magnesium precipitate hot start method provides a significantly improved

10 specificity of PCR target amplification compared to the standard PCR reactions. The temperature at which the magnesium ions are released is achieved during the first cycle of the PCR amplification in the thermal cycler thereby eliminating any extra steps and need for additional reagents.

In addition to the applicability of this invention in standard PCR reactions, the

15 formation of a precipitate containing magnesium could be utilized in "long and accurate" PCR. Specifically, "long and accurate" PCR could conveniently be provided the advantages of a hot start without tedious extra care or steps thus resulting in increased reliability and efficiency of human STR typing and multiplex PCR. Such long and accurate PCR is described in Barnes, *Proc. Natl. Acad. Sci. USA,* 91:2216- 2220 (1994)

20 and in U.S. Pat. No. 5,436,149. Furthermore, the magnesium precipitate hot start method can be applied in the RT-PCR reaction, wherein the desired RNA sequence is first reverse transcribed into the cDNA, and then amplified by PCR. Besides a greater specificity of product amplification, the magnesium precipitate hot start method possesses other beneficial attributes such as the ease of manipulation, the little extra time necessary to

25 perform it, and the inexpensive reagents required.

Further, the present invention may be utilized in any process which requires amplification. For instance, the formation of a precipitate containing magnesium may be utilized in processes of in vivo footprinting which use a DNA polymerase to amplify the DNA. In general, analysis of the interaction of proteins with either DNA or RNA by in

5 vivo footprinting involves first modifying the nucleic acids by the footprinting reagent in situ. Footprinting reagents are chosen based on how extensively the reactivity of a nucleic acid toward the modifying agent is altered upon interaction with the binding protein of interest. The modifications are then visualized (i.e., the analysis of the reactivity of each nucleotide of the sequence of interest) usually by PCR. See Grange et al., *Methods,* (1997)
10 11:151-63. Accordingly, LM-PCR is utilized to visualize modifications in DNA molecules and RL-PCR is utilized to visualize modifications in RNA molecules. Both LM-PCR and RL- PCR involve ligating a linker to the unknown 5'-ends resulting from the in vivo footprinting analysis and exponentially amplifying the region of interest. In LM-PCR, a blunt double-stranded end is created using a gene specific primer and a DNA polymerase.
15 Then a partially double--stranded DNA linker with one blunt end is ligated to the blunt ends using a DNA ligase. The strand onto which the linker has been ligated will then serve as a template for PCR amplification. Similarly, in RL-PCR, a single stranded RNA linker is ligated to the 5' P-ends of all RNA molecules using a RNA ligase. Then a CDNA copy of the sequence of interest is synthesized using a reverse transcriptase which results in
20 generating templates for PCR amplification. Lastly, amplified products from LM-PCR and RL-PCR are then labeled and sequenced for analysis.

A precipitate containing magnesium is also applicable to processes of primer directed mutagenesis using DNA polymerases to amplify the mutated nucleic acid sequences having substitution mutations within the target DNA sequence. The process of
25 primer directed mutagenesis comprises contacting a nucleic acid sequence with two mutated primers, where each mutation is a mismatch when compared to the template sequence; amplifying using DNA polymerase; and allowing the amplified products to reanneal. The resulting nucleic acid molecules amplified using these mismatched mutated

5 primers have mismatched bases and have a double-stranded region containing a mutant strand. See Innis et al., "PCR Protocols", Academic Press, 1990, pp. 177-183.

The use of a precipitate containing magnesium is further applicable to processes of DNA restriction digest filling using DNA polymerases to amplify the DNA. DNA polymerases are used in restriction digest filling to extend the 3' ends resulting from
10 digestion with restriction enzymes for the purpose of producing 5'-sticky ends. The process comprises separating the digested DNA strands; contacting each 3' end of the separated nucleic acid molecules with oligodeoxyribonuclotide primers; extending the 3' ends using DNA polymerase to create blunt ends; and allowing the DNA strands with the newly synthesized 3' ends to reanneal to its complementary strand.

15 In a further aspect, the present application provides kits for the use of the magnesium precipitate hot start in PCR reactions. Accordingly, a reagent containing a pre-formed precipitate containing a source of phosphate ions and a source of magnesium ions and other PCR reagents are provided in the form of a test kit, that is, in a packaged collection or combination as appropriate for the needs of the user. Instructions for utilizing
20 a precipitate containing magnesium in a process of amplifying a target nucleic acid are provided in the kits of the present invention. Preferably, the kit provides a pre-formed precipitate containing magnesium ions and instructions for utilizing the precipitate to amplify a nucleic acid sequence. In another embodiment, the kit comprises a container comprising a source of phosphate ions and a container comprising a source of magnesium
25 ions, wherein combining two sources at a temperature of below 34° C. results in the formation of a precipitate, and instructions for using the source of phosphate ions and a source of magnesium ions to amplify a target nucleic acid. The kit can of course include appropriate packaging, containers, labeling, and buffers for amplifying a target nucleic acid. In another preferred embodiment, the kit also includes a DNA polymerase and more

5 preferably, deoxyribonucleoside triphosphates. In another embodiment, a kit comprises instructions for using a source of phosphate ions and a source of magnesium ions in amplifying a target nucleic acid sequence.

All publications, patents, patent applications or other references cited in this 10 application are herein incorporated by reference in their entirety as if each individual publication, patent, patent application or reference are specifically and individually indicated to be incorporated by reference.

The following examples illustrate the invention.

EXAMPLES

15 **Example 1**

The Effect of Phosphoric Acid Concentration on PCR Product Amplification

The human tissue plasminogen activator (t-PA) gene was amplified using different hot start PCR methods. The standard PCR program for this gene included holding the reaction for 3 minutes at 68° C., after which 40 cycles were performed with the following 20 parameters: 93° C. for 50 seconds, 67° C. for 40 seconds, and 68° C. for 5 minutes. The primers for the amplification of human t-PA gene were:

t-PA forward 7: GGAAGTACAGCTCAGAGTTCTGCAGCACCCCTGC (SEQ. ID. NO. 1)

t-PA reverse 7.5: TGGGATTATAGACACGAGCCACTGCACCTGGCCC (SEQ. 25 ID. NO. 2).

The choice of primers follows Degen et al., *J. Biol. Chem.,* 261(15):6972-6985 (1986) and Flynn S. (unpublished, 2000). The enzyme that was used was KlentaqLA. The expected size of the product was 880 bp. In addition to the standard protocol, all the samples in FIG. 1 were incubated at 30° C. for 30 minutes prior to the first step of the

5 PCR reaction (warm start). This step was included as a control for the efficacy of the magnesium precipitate hot start.

The manual hot start was achieved by adding 5 ul of 35 mM magnesium chloride after 30 seconds at 68° C. (hot start is denoted in FIGS. 1 and 2 with a letter H), whereas magnesium chloride was added at room temperature to warm start reactions. The warm
10 start involving the additional step of incubation at 30° C. for 30 minutes was denoted with a letter W in FIG. 1. B denotes a standard or bench PCR protocol, wherein all PCR reagents are mixed and are directly transferred to the cycler. The superscript designation in FIGS. 1 and 2 denotes the concentration of phosphoric acid in the reaction, which was included in the TAT buffer. For the magnesium precipitate hot start, the appropriate TAT
15 buffer (4.5 ul) was incubated with 35 mM magnesium chloride (5 ul)for at least 10 minutes at room temperature to allow precipitate to form, after which the master mix was added to the tubes. The mastermix contained the following:

10 mM each dNTP (each 0.5 ul/rxn)

10 ul tPA forward primer (1 ul/rxn)

20 10 ul tPA reverse primer (1 ul/rxn)

10 ng/ul human DNA (0.05 ul/rxn)

5 M Betaine (13 ul/rxn)

100% DMSO (0.75 ul/rxn), and

KlentaqLA Mix (5 ul/rxn), wherein KlentaqLA Mix consisted of 2.25 ul
25 KlentaqLA, 15 ul TAT, and 132.75 ul dH 2 O,

and sufficient volume of dH 2 O so that the total volume of the reaction is 50 ul.

The reactions were then run in the RoboCycler40 thermocycler at the previously specified conditions. The amplification products were observed on the agarose gel stained with ethidium bromide.

5 In FIG. 1 , the concentration of phosphoric acid ranged from 3 mM to 7 mM, whereas in FIG. 2 it ranged from 5 mM to 19 mM. As can be seen from the Figures, the magnesium precipitate hot start was as efficient as the manual hot start. The optimal concentration range of phosphoric acid in the manual hot start is between 5 mM and 7 mM, however the method still works well at concentrations between 3 mM and 13 mM.

10 The procedure as specified above was also performed with incubations taking place at 34° C. and 37° C.; however, the magnesium precipitate PCR reactions did not work as effectively as manual hot start reactions at these temperatures (data not shown).

Example 2

The Use of Different Polymerases in the Magnesium Phosphate Hot Start Method

15 The human t-PA gene was amplified using the standard PCR protocol described in Example 1. In addition, TaqLA Mix that was also used in this Example 2 consisted of 5 ul TaqLA, 5 ul TAT, and 40 ul dH 2 O. The reactions were run in duplicate, and each of the hot start (H), bench start (B) or bench start with phosphoric acid (i.e. B^{5} or the magnesium precipitate hot start method) was performed with both KlentaqLA and TaqLA (for either 20 enzyme, 5 ul of the enzyme mix was used per reaction). The bands in FIG. 3 depict the products of these reactions. The bench start yielded very light bands, indicating that the specificity of the reaction was suboptimal whereas both the manual and the magnesium precipitate hot start reactions resulted in high amplification of the product with both KlentaqLA and TaqLA. The data suggest that multiple DNA polymerases can be utilized 25 to perform magnesium precipitate hot start PCR with similar efficiencies.

Example 3

The Effect of Varying Phosphate Comprising Compounds on PCR Product Amplification

5 Human t-PA gene was amplified with KlentaqLA using the standard protocol from Example 1 either utilizing the manual hot start method (magnesium chloride added at 68° C. after 30 seconds) or the magnesium precipitate hot start method. For the magnesium precipitate hot start, TAT buffers containing different phosphate comprising compounds were formulated. The following compounds were used: phosphoric acid (H_3PO_4),
10 potassium phosphate (KH_2PO_4), sodium phosphate (NaH_2PO_4), and MDP (methylenediphosphonic acid, ($CH_6O_6P_2$), each at a final concentration of 5 mM in the reaction. The resulting pHs were:

8.8-TAT+5 mM phosphoric acid

9.1-TAT+5 mM KH_2PO_4

15 9.2-TAT+5 mM NaH_2PO_4

8.7-TAT+5 mM MDP.

As FIG. 5 shows, the reactions performed with TAT buffer generated well amplified products only with the manual hot start whereas bench start resulted in low amplification. The reactions containing phosphoric acid and KH_2PO_4 yielded bands on
20 the gel, however the phosphoric acid was superior in performance compared to KH_2PO_4. The reactions with NaH_2PO_4 generated hardly any amplifications products, and there were no products in the reactions with MDP indicating that these two compounds are not the adequate choices for performing hot start PCR reactions. However, some optimization of KH_2PO_4 might render it suitable for use in magnesium precipitate hot start PCR. At
25 present, the phosphoric acid is the most adequate source of phosphate ions for the magnesium precipitate hot start.

Example 4

The Effect of Magnesium Chloride Concentration on Precipitate Formation

5 The human t-PA gene was amplified using the standard protocol described in Example 1. FIG. 6 shows the effect of varying magnesium chloride concentration in manual and hot start PCRs. The DNA polymerase used in both methods was TaqLA. The manual hot start reactions were performed with TAT buffer while the magnesium precipitate hot start PCR reactions were performed with TAT5. The concentrations of
10 magnesium chloride that were tested included 0.5 mM, 1 mM, 2 mM, 4 mM, and 8 mM. The magnesium chloride concentration of 0.5 mM resulted in no amplification of the product in either manual or magnesium precipitate hot start and at concentrations of 2 mM and 4 mM both PCR methods worked well. At the high concentration of magnesium (8 mM) the manual hot start worked better than the magnesium precipitate method. This was
15 expected, since at high concentrations of magnesium, there was not enough phosphate in the solution to sequester a significant proportion of magnesium ions, thereby allowing DNA polymerase to extend the primers. However, the concentration curve of magnesium indicated that the magnesium precipitate hot start is effective over the range of magnesium concentrations commonly used in PCR.

20 **Example 5**

The Time of Incubation Influences the Precipitate Formation

Human t-PA gene was amplified using the standard protocol described in Example 1. The manual hot start (H) and the bench start (B) were performed with TAT buffer. The hot start resulted in a significant amplification of the t-PA gene, whereas the bench start
25 yielded a minimal band. For the magnesium precipitate hot start reactions, TAT5 buffer was incubated with magnesium chloride for different lengths of time prior to the addition of the mastermix. All the reactions mentioned in this example and depicted in FIG. 7 were performed with KlentaqLA. The reactions were done in duplicate, and the incubation times were 15 minutes, 10 minutes, 5 minutes, 2 minutes, and 0 minutes. It can be seen

5 from the figure that no incubation time or 2 minutes are not long enough for the precipitate to form and simulate a hot start. At 5 minutes, there is a slight amplification of the product, however the best amplification is observed after 15 minutes of incubation. This data suggests that in order for the magnesium precipitate hot start to function properly a source of phosphate and a source of magnesium need to be incubated for at least 5 10 minutes, with the amplification significantly improving after 10- 15 minutes of incubation.

Example 6

The Universality of the Magnesium Precipitate Hot Start Method

Examples 1-5 have shown that the magnesium precipitate hot start method works efficiently with the human t-PA gene. In order to show that this method is not limited to 15 certain genes, the same method was utilized to amplify a viral gene. The gene utilized was HIV-1 gag. The primers for the amplification of HIV-1 gag were:

SK 38: ATAATCCACCTATCCCAGTAGGAGAAAT (SEQ. ID. NO. 3)

SK39: TTTGGTCCTTGTCTTATGTCCAGAATGC (SEQ. ID. NO. 4).

These primers and the HIV-1 DNA were supplied by Applied Biosystem's 20 GeneAmplimer HIV-1 Control Reagents. The use of the primers is disclosed in Ou et al., *Science* 239: 295-297 (1998).

The PCR program for amplification of this gene included holding the reaction at 68° C. for 3 minutes followed by 42 cycles with the following conditions: 95° C. for 40 seconds, 52° C. for 40 seconds, and 68° C. for 1 minute. The mastermix contained:

25 10 mM each DNTP (each 0.5 ul/rxn)

25 um SK 38 primer (0.5 ul/rxn)

25 uM SK 39 primer (0.5 ul/rxn)

25 copies/ul HIV-1 DNA+100 ng/ul denatured human DNA (1 ul/rxn)

5 Klentaq1 Mix (5 ul/rxn), wherein said mix consisted of 2 ul Klentaq1, 20 ul TAT, and 178 ul dH 2 O,

and sufficient dH 2 O for the total reaction volume of 50 ul.

For the magnesium precipitate hot start, 5 ul of 35 mM magnesium chloride was added to a reaction tube containing 4.5 ul TAT5 and incubated for at least 10 minutes at 10 room temperature to allow the precipitate to form. The mastermix was then prepared and added to the tubes for both manual and magnesium precipitate hot starts (magnesium was excluded for the manual hot start, and then added to the appropriate tubes in the cycler after 30 seconds at 68° C.).

The results of the FIG. 8 show that the bench start was very non- specific, generating an 15 incorrect band whereas the magnesium precipitate hot start, similar to the manual hot start was very specific and resulted in a significant amplification of the gag gene.

Example 7

Various Magnesium Containing Compounds can Result in Precipitate Formation

Different magnesium containing compounds were tested for the ability to form a 20 precipitate with phosphoric acid and to facilitate the magnesium precipitate hot start method. The compounds tested were magnesium chloride, magnesium sulfate, magnesium hydroxide, and magnesium carbonate. As shown in Examples 1-6, magnesium chloride was previously tested and shown to work in the magnesium hot start methods. Thus, magnesium chloride was utilized in this Example 7 as a positive control. The PCR 25 program and the protocols for performing bench start, manual start and magnesium precipitate hot starts are described in the previous examples. As seen in FIGS. 4 *a* and **4** *b,* the bench start reactions performed with all four magnesium containing compounds yielded the bands of incorrect size. The manual hot start reactions worked well with magnesium chloride, magnesium hydroxide, and magnesium carbonate, whereas the

5 manual hot start performed with magnesium sulfate generated very little product. The magnesium precipitate hot start method worked efficiently with all four magnesium containing compounds (magnesium chloride, magnesium sulfate, magnesium hydroxide, and magnesium carbonate). This could prove to be useful in PCR reactions wherein the DNA polymerases require magnesium in a form other than the most commonly used
10 magnesium chloride.

Example 8

The Effect of Ammonium on Precipitate Formation

The gene that was amplified in this Example 8 was HIV-1 gag and its amplification was performed according to the protocol specified in Example 6. The
15 changes from that protocol include the use of 50 copies of HIV genome instead of 25 copies and the cycling in the RoboCycler was performed 44 times rather than 42 times. The reactions that were prepared and run in the cycler were the manual hot start, the bench start, and reactions that omitted either ammonium sulfate or both Tris and ammonium sulfate during the incubation step. In the reaction where both ammonium sulfate and Tris
20 were withheld, the mixture of 100 ul 35 mM magnesium chloride and 100 ul of 50 mM phosphoric acid was incubated for 24 hours at room temperature and then for additional 12 hours at 4° C. For the reaction where only ammonium sulfate was withheld, 50 ul of 1 M Tris Base was added to the mixture of the same composition as above and incubated at the same conditions. Prior to the transfer into the thermal cycler, 10 ul of the first mixture and
25 12.5 ul of the second mixture were added to the tubes. The appropriate missing reagents were then added to both tubes and the reactions were subjected to the amplification in the cycler. It can be seen from the FIG. 9 that the manual hot start generated a well amplified correct product whereas the bench start generated the product of the incorrect size. The two reactions that excluded either ammonium sulfate or both Tris and ammonium sulfate

5 generated the amplification products, however the amplification was not as optimal as when both Tris and ammonium sulfate are also provided in the buffer during the precipitate formation.

Example 9

Concentration Curve of Ammonium Phosphate in Magnesium Precipitate Hot Start

10 Reactions

Cryptosporidium parvum heat shock protein homolog gene (hsp70) was amplified using the following PCR program: 68° C. for 3 minutes, followed by 42 cycles of 95° C. for 40 seconds, 58° C. for 1 minute, and 68° C. for 2 minutes, and the results are shown in FIG. **10** . The primers used to amplify this gene were:

15 CPHSPT2F: TCCTCTGCCGTACAGGATCTCTTA (SEQ. ID. NO. 5) and CPHSPT2R: TGCTGCTCTTACCAGTACTCTTATCA (SEQ. ID. NO. 6).
See Di Giovanni et al., "Real Time Quantitative PCR Detection of Intact and Infectious *Cryptosporidium parvum* Oocysts for Water Industry Applications", Abstracts of the 100th General Meeting of the American Society for Microbiology, 2000.

20 The mastermix consisted of:

10 mM each DNTP (0.5 ul each dNTP/rxn)

10 uM CPHSPT2F (1 ul/rxn)

10 uM CPHSPT2R (1 ul/rxn)

1 pg/ul *Cryptosporidium parvum* DNA (0.1 ul/rxn)

25 200 ng/ul denatured Calf Thymus DNA (2 ul/rxn)

Klentaq1 Mix (5 ul/rxn),

and sufficient volume of dH 2 O so that the total volume of the reaction is 50 ul.

The manual hot start and the bench start reactions were performed as in the previous examples. H [5] denotes the manual hot start reaction, wherein the TAT buffer

5 contained 5 mM ammonium phosphate ($(NH_4)_2HPO_4$) as the source of phosphate ions. In the magnesium precipitate hot start reactions, the amount of ammonium phosphate was varied in such way that only the phosphate concentration was effectively changed. The concentration of ammonium ions was kept constant by decreasing the amount of ammonium sulfate in the TAT buffer. The TAT buffers modified in this manner are

10 marked in the FIG. 10 as TA'T. All the modified buffers had the same pH of 9.2.

As can be seen from FIG. 10 , both forms of manual hot start worked well with or without ammonium phosphate (H^5 and H, respectively) whereas the bench start without ammonium phosphate resulted in the amplification of two incorrect bands. The same incorrect bands were observed in the magnesium precipitate hot start method with 1 mM

15 ammonium phosphate. However, 3 mM and 5 mM concentrations of ammonium phosphate in magnesium precipitate hot start reactions gave rise to significant product amplification. Therefore, ammonium phosphate can successfully be applied in magnesium precipitate hot start PCR as a source of phosphate ions. Furthermore, ammonium phosphate possesses a different range of phosphate concentrations at which magnesium

20 precipitate hot start is functional compared to the phosphoric acid. The ability to use different effective concentration ranges of different phosphate containing compounds allows for broad application of magnesium precipitate hot start method in PCR reactions. These ranges of concentrations can be determined by a skilled artisan using the methods disclosed herein.

25 It is to be understood that the present invention has been described in detail by way of illustration and example in order to acquaint others skilled in the art with the invention, its principles, and its practical application. Further, the specific embodiments of the present invention as set forth are not intended as being exhaustive or limiting of the invention, and that many alternatives, modifications, and variations will be apparent to

5 those skilled in the art in light of the foregoing examples and detailed description. Accordingly, this invention is intended to embrace all such alternatives, modifications, and variations that fall within the spirit and scope of the following claims. While some of the examples and descriptions above include some conclusions about the way the invention may function, the inventors do not intend to be bound by those conclusions and functions,
10 but puts them forth only as possible explanations.

5 **We claim:**

1. A process for synthesizing a nucleic acid extension product, said process comprising:

a. forming or obtaining a reagent comprising a source of magnesium ions and a source of phosphate ions, wherein the source of magnesium ions and the source
10 of phosphate ions form a precipitate at a temperature below 34° C.;

b. making a mixture comprising the reagent of step (a), wherein said magnesium ions are contained in said precipitate, a DNA polymerase, deoxyribonucleoside triphosphates, primers and a target nucleic acid sequence;

c. releasing the magnesium ions from the precipitate at a temperature
15 above 34° C. thereby activating the DNA polymerase;

d. if the nucleic acid is double stranded, separating the strands and denaturing intrastrand structures;

e. annealing the primers to the target nucleic acid sequence at an appropriate temperature; and

20 f. synthesizing an extension product of each primer using the activated DNA polymerase, wherein the primer extension product is complementary to the target nucleic acid DNA strand.

2. The process of claim 1 further comprising:

a. separating the primer extension products from the templates on
25 which they are synthesized to produce single-stranded molecules; and

b. repeating steps (f) and (g) at least once.

3. The process of claim 1 , wherein the precipitate comprises magnesium and phosphate.

5 4. The process of claim 3 , wherein the reagent further comprises ammonium sulfate and Tris(hydroxymethyl)aminomethane.

5. The process of claim 1 , wherein the magnesium ions are released from the precipitate in step (c) by heating the mixture to alternating temperatures within the range of between 50° C. and 95° C.

10 6. The process of claim 1 wherein the source of phosphate ions in the reagent comprises phosphoric acid, potassium phosphate or ammonium phosphate.

7. The process of claim 6 wherein the reagent comprises phosphoric acid at a concentration of about 3 to 13 mM.

8. The process of claim 6 wherein the reagent comprises ammonium

15 phosphate at a concentration of about 2 to 6 mM.

9. The process of claim 6 wherein the reagent has a pH above 7.

10. The process of claim 6 wherein the reagent further comprises Tris at a concentration of about 50 mM.

11. The process of claim 6 wherein the reagent further comprises ammonium

20 sulfate at a concentration of about 16 mM.

12. The process of claim 1 wherein the source of magnesium ions in the reagent comprises magnesium chloride, magnesium hydroxide, magnesium carbonate or magnesium sulfate.

13. The process of claim 1 wherein if the target nucleic acid sequence is a RNA

25 sequence, first reverse transcribing the RNA into cDNA.

14. The process of claim 1 wherein the DNA polymerase is TaqLA, KlentaqLA or Klentaq1.

15. The process of claim 14 wherein the source of phosphate ions is phosphoric acid and the source of magnesium ions is magnesium chloride.

5 16. The process of claim 14 wherein the magnesium ions are released from the precipitate in step (c) by heating the mixture to alternating temperatures within the range of between 50° C. and 95° C.

17. A method of molecular manipulation of a nucleic acid sequence with an enzyme, said method comprising:

10 a. forming or obtaining a reagent comprising a source of magnesium ions and a source of phosphate ions, wherein the source of magnesium ions and the source of phosphate ions form a precipitate at a temperature below a temperature at which specific enzymatic manipulation occurs;

b. making a mixture comprising a precipitate of the reagent of step (a),
15 a magnesium dependant enzyme and the nucleic acid sequence;

c. releasing the magnesium ions into the mixture thereby activating said enzyme; and

d. allowing the enzyme to catalyze the manipulation of said nucleic acid.

20 18. A method of cleaving a nucleic acid sequence with a restriction endonuclease, said method comprising:

a. forming or obtaining a reagent comprising a source of magnesium ions and a source of phosphate ions, wherein the source of magnesium ions and the source of phosphate ions form a precipitate at a temperature below a temperature at which
25 specific cleaving occurs;

b. making a mixture comprising a precipitate of the reagent of step (a), a restriction endonuclease and the nucleic acid sequence comprising a restriction site for said restriction endonuclease;

5 c. releasing the magnesium ions into the mixture thereby activating the restriction endonuclease;

d. allowing the restriction endonuclease to recognize and bind to the recognition sequence of the nucleic acid sequence; and

e. cleaving said nucleic acid.

10 19. A method for reverse transcribing an RNA template, said method comprising:

a. forming or obtaining a reagent comprising a source of magnesium ions and a source of phosphate ions, wherein the source of magnesium ions and the source of phosphate ions form a precipitate at a temperature below a temperature at which
15 specific reverse transcription occurs;

b. making a mixture comprising a precipitate of the reagent of step (a), said RNA template, an oligonucleotide primer, which primer is sufficiently complementary to said RNA template to hybridize therewith, and reverse transcriptase in the presence of all four deoxyribonucleoside triphosphates;

20 c. releasing the magnesium ions into the mixture thereby activating the reverse transcriptase; and

d. allowing said primer to hybridize to said RNA template and said reverse transcriptase to catalyze the polymerization of said deoxyribonucleoside triphosphates to provide cDNA complementary to said RNA template.

25 20. A method of ligating nucleic acid sequences with a thermostable DNA ligase, said method comprising:

a. forming or obtaining a reagent comprising a source of magnesium ions and a source of phosphate ions, wherein the source of magnesium ions and the source

5 of phosphate ions form a precipitate at a temperature below a temperature at which non-specific ligation occurs;

b. making a mixture comprising a precipitate of the reagent of step (a), the DNA ligase and the nucleic acid sequences;

c. releasing the magnesium ions into the mixture thereby activating the
10 thermostable DNA ligase; and

d. allowing the thermostable DNA ligase to ligate nucleic acid sequences.

21. The method of claim 17 wherein the source of magnesium ions is selected from the group consisting of magnesium chloride, magnesium hydroxide, magnesium
15 carbonate and magnesium sulfate.

22. The method of claim 18 wherein the source of magnesium ions is selected from the group consisting of magnesium chloride, magnesium hydroxide, magnesium carbonate and magnesium sulfate.

23. The method of claim 19 wherein the source of magnesium ions is selected
20 from the group consisting of magnesium chloride, magnesium hydroxide, magnesium carbonate and magnesium sulfate.

24. The method of claim 20 wherein the source of magnesium ions is selected from the group consisting of magnesium chloride, magnesium hydroxide, magnesium carbonate and magnesium sulfate.

25 25. The method of claim 17 wherein the source of phosphate ions is selected from the group consisting of phosphoric acid, potassium phosphate, and ammonium phosphate.

5 26. The method of claim 18 wherein the source of phosphate ions is selected from the group consisting of phosphoric acid, potassium phosphate, and ammonium phosphate.

27. The method of claim 19 wherein the source of phosphate ions is selected from the group consisting of phosphoric acid, potassium phosphate, and ammonium
10 phosphate.

28. The method of claim 20 wherein the source of phosphate ions is selected from the group consisting of phosphoric acid, potassium phosphate, and ammonium phosphate.

29. The method of claim 17 wherein the source of magnesium ions is
15 magnesium chloride and the source of phosphate ions is phosphoric acid.

30. The method of claim 18 wherein the source of magnesium ions is magnesium chloride and the source of phosphate ions is phosphoric acid.

31. The method of claim 19 wherein the source of magnesium ions is magnesium chloride and the source of phosphate ions is phosphoric acid.

20 32. The method of claim 20 wherein the source of magnesium ions is magnesium chloride and the source of phosphate ions is phosphoric acid.

33. The method of claim 17 wherein releasing the magnesium ions into the mixture comprises heating reagents to a temperature standard for the enzyme manipulation.

25 34. The method of claim 18 wherein releasing the magnesium ions into the mixture comprises heating reagents to a temperature standard for cleaving a nucleic acid sequence.

5 35. The method of claim 19 wherein releasing the magnesium ions into the mixture comprises heating reagents to a temperature standard for reverse transcribing an RNA template.

36. The method of claim 20 wherein releasing the magnesium ions into the mixture comprises heating reagents to a temperature standard for the ligating of the
10 nucleic acid with a thermostable DNA ligase.

37. The method of claim 17 wherein the source of magnesium ions and the source of phosphate ions form a precipitate at a temperature of below 34°C.

38. The method of claim 18 wherein the source of magnesium ions and the source of phosphate ions form a precipitate at a temperature of below 34°C.

15 39. The method of claim 19 wherein the source of magnesium ions and the source of phosphate ions form a precipitate at a temperature of below 34°C.

40. The method of claim 20 wherein the source of magnesium ions and the source of phosphate ions form a precipitate at a temperature of below 34°C.

41. The method of claim 37 wherein the releasing of the magnesium ions
20 comprises heating the reagents to a temperature of above 30°C.

42. The method of claim 38 wherein the releasing of the magnesium ions comprises heating the reagents to a temperature of above 30°C.

43. The method of claim 39 wherein the releasing of the magnesium ions comprises heating the reagents to a temperature of above 30°C.

25 44. The method of claim 40 wherein the releasing of the magnesium ions comprises heating the reagents to a temperature of above 30°C.

45. A kit for molecular manipulation of a nucleic acid sequence with an enzyme, said kit comprising:

a. a container containing a source of magnesium ions;

5 b. a container containing a source of phosphate ions, wherein said source of magnesium ions and said source of phosphate ions form a precipitate containing magnesium when combined at temperatures below the temperature which specific molecular manipulation occurs; and

c. instructions for performing said molecular manipulations.

10 46. A kit for cleaving of a nucleic acid sequence with an enzyme, said kit comprising:

a. a container containing a source of magnesium ions;

b. a container containing a source of phosphate ions, wherein said source of magnesium ions and said source of phosphate ions form a precipitate containing 15 magnesium when combined at temperatures below the temperature at which non-specific cleaving of the nucleic acid occurs; and

c. instructions for performing said cleaving.

47. A kit for reverse transcribing an RNA template with an enzyme, said kit comprising:

20 a. a container containing a source of magnesium ions;

b. a container containing a source of phosphate ions, wherein said source of magnesium ions and said source of phosphate ions form a precipitate containing magnesium when combined at temperatures below the temperature at which non-specific reverse transcription occurs; and

25 c. instructions for performing said reverse transcription.

48. A kit for ligating nucleic acid sequences with an enzyme, said kit comprising:

a. a container containing a source of magnesium ions;

5 b. a container containing a source of phosphate ions, wherein said source of magnesium ions and said source of phosphate ions form a precipitate containing magnesium when combined at temperatures below the temperature which specific molecular manipulation occurs; and

c. instructions for performing said ligation.

10 49. A kit for molecular manipulation of a nucleic acid sequence with an enzyme, said kit comprising:

a. a container containing a reagent comprising a precipitate containing magnesium;

b. instructions for using the precipitate containing magnesium for

15 performing said molecular manipulations.

50. A kit for amplifying a target nucleic acid, said kit comprising:

a. a container comprising a source of phosphate ions and a container comprising a source of magnesium ions, wherein combining the source of magnesium ions and the source of phosphate ions form a precipitate at a temperature below 34°C; and

20 b. instructions for using the source of phosphate ions and the source of magnesium ions in amplification of the target nucleic acid.

51. The kit of claim 50 wherein the source of phosphate ions is phosphoric acid and the source of magnesium ions is magnesium chloride.

52. The kit of claim 51 wherein said kit further comprises a container

25 comprising a DNA polymerase.

53. The kit of claim 52 wherein the DNA polymerase is TaqLA, Klentaql, KlentaqLA, Pfu, Deep Vent or Tth.

54. The kit of claim 53 wherein said kit further comprises a container comprising deoxyribonucleoside triphosphates.

5 55. The kit of claim 51 wherein said kit further comprises a container comprising a mixture of at least two DNA polymerases selected from the group consisting of TaqLA, Klentaql, KlentaqLA, Pfu, Deep Vent and Tth.

56. The kit of claim 55 wherein said kit further comprises a container comprising deoxyribonucleoside triphosphates.

10 57. A kit for amplifying a target nucleic acid, said kit comprising:

a. a container comprising a reagent comprising a precipitate comprising a magnesium salt; and

b. instructions for using the precipitate to amplify the target nucleic acid.

15 58. The kit of claim 57 wherein said kit further comprises a DNA polymerase.

59. The kit of claim 58 wherein said DNA polymerase is TaqLA, Klentaql, KlentaqLA, Pfu, Deep Vent or Tth.

60. The kit of claim 58 wherein said kit further comprises a container comprising deoxyribonucleoside triphosphates.

20 61. The kit of claim 57 wherein said kit further comprises a container comprising a mixture of at least two DNA polymerases selected from the group consisting of TaqLA, Klentaql, KlentaqLA, Pfu, Deep Vent and Tth.

62. The kit of claim 61 wherein said kit further comprises a container comprising deoxyribonucleoside triphosphates.

25 63. A kit for amplifying a target nucleic acid, said kit comprising instructions for using a source of phosphate ions and a source of magnesium ions in amplification of a target nucleic acid.

FIG. 1

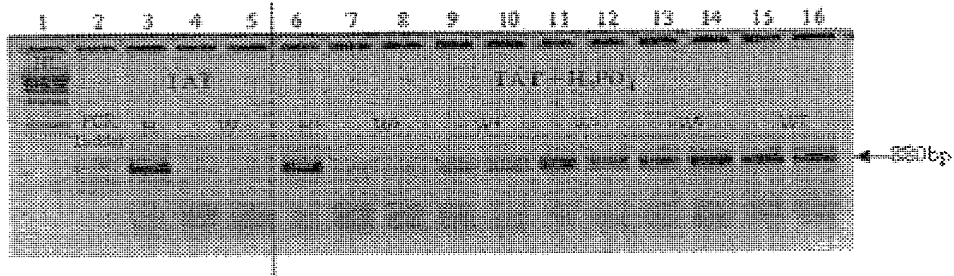


FIG. 2

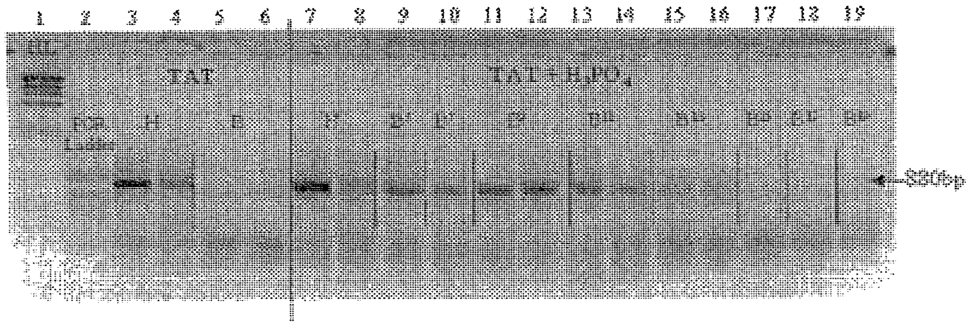


FIG. 3

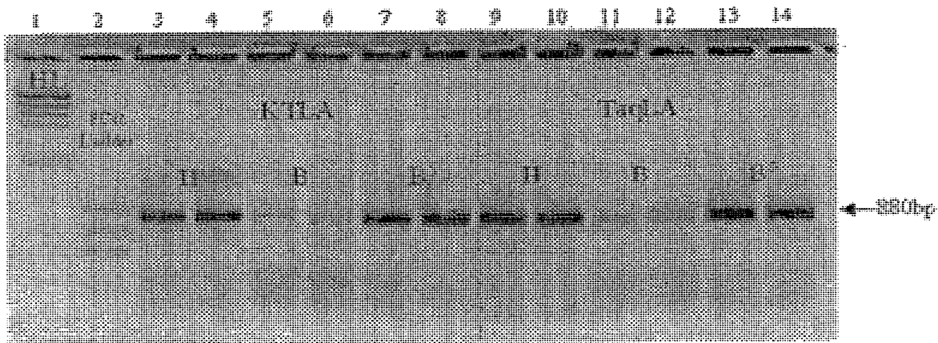


FIG. 4A

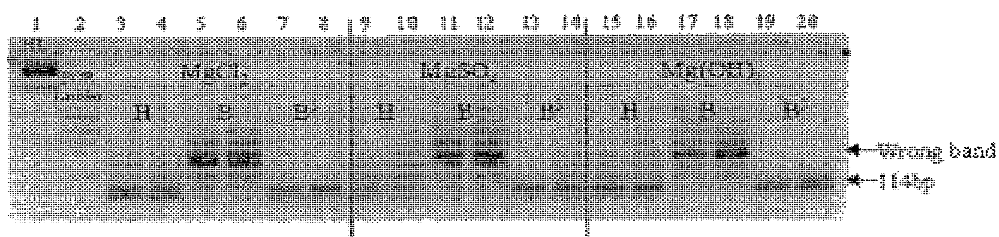


FIG. 4B

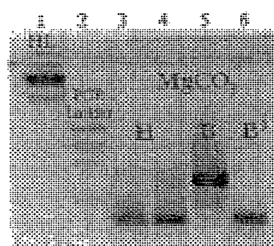


FIG. 5A

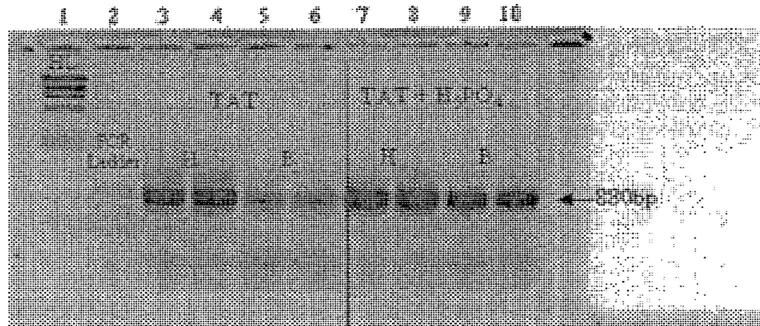


FIG. 5B

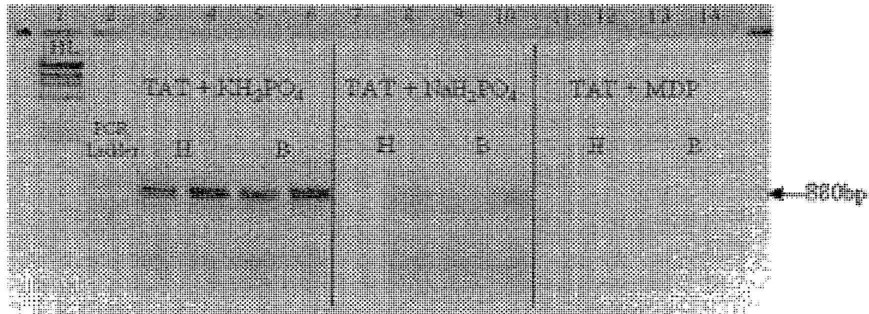


FIG. 6

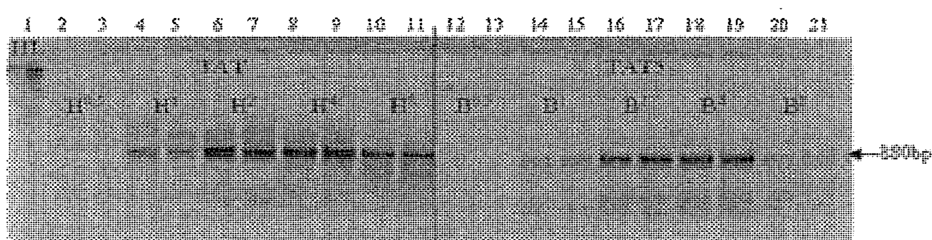


FIG. 7

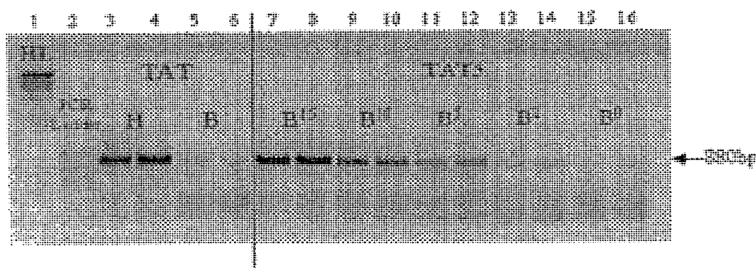


FIG. 8

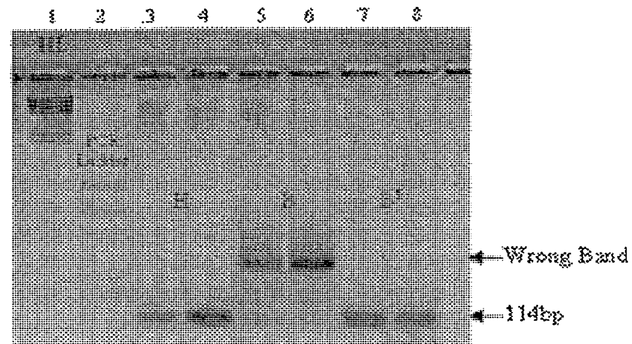


FIG. 9

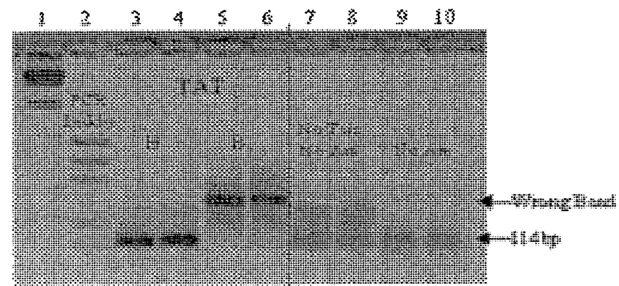
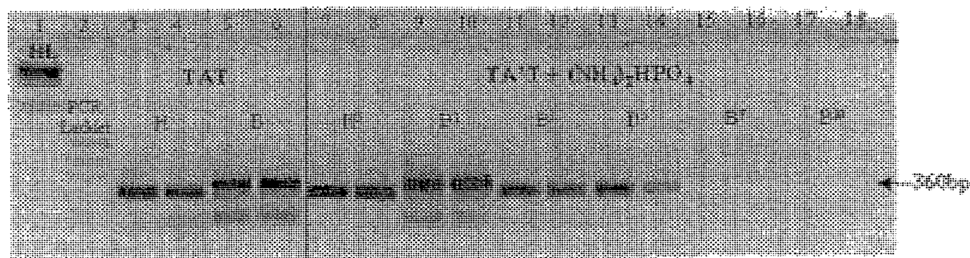


FIG. 10



(12) INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(19) World Intellectual Property Organization
International Bureau

(43) International Publication Date
13 February 2003 (13.02.2003)

PCT

(10) International Publication Number
WO 03/012066 A3

(51) International Patent Classification[7]: C12Q 1/68, C12P 19/34, C07H 21/04

(21) International Application Number: PCT/US02/24533

(22) International Filing Date: 2 August 2002 (02.08.2002)

(25) Filing Language: English

(26) Publication Language: English

(30) Priority Data:

60/309,646	2 August 2001 (02.08.2001)	US
09/920,872	2 August 2001 (02.08.2001)	US
10/091,784	6 March 2002 (06.03.2002)	US

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(81) Designated States *(national)*: AE, AG, AL, AM, AT, AU, AZ, BA, BB, BG, BR, BY, BZ, CA, CH, CN, CO, CR, CU, CZ, DE, DK, DM, DZ, EC, EE, ES, FI, GB, GD, GE, GH, GM, HR, HU, ID, IL, IN, IS, JP, KE, KG, KP, KR, KZ, LC, LK, LR, LS, LT, LU, LV, MA, MD, MG, MK, MN, MW, MX, MZ, NO, NZ, OM, PH, PL, PT, RO, RU, SD, SE, SG, SI, SK, SL, TJ, TM, TN, TR, TT, TZ, UA, UG, US, UZ, VN, YU, ZA, ZM, ZW.

(84) Designated States *(regional)*: ARIPO patent (GH, GM, KE, LS, MW, MZ, SD, SL, SZ, TZ, UG, ZM, ZW), Eurasian patent (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European patent (AT, BE, BG, CH, CY, CZ, DE, DK, EE, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE, SK, TR), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, GQ, GW, ML, MR, NE, SN, TD, TG).

Published:
— *with international search report*

(88) Date of publication of the international search report: 4 December 2003

For two-letter codes and other abbreviations, refer to the "Guidance Notes on Codes and Abbreviations" appearing at the beginning of each regular issue of the PCT Gazette.

WO 03/012066 A3

(54) **Title:** MAGNESIUM PRECIPITATE HOT START METHOD FOR MOLECULAR MANIPULATION OF NUCLEIC ACIDS

(57) **Abstract:** The present invention provides methods of performing enzymatic reactions, including PCR, which require the use of magnesium dependent enzymes, including restriction endonucleases, ligases, and reverse transcriptases. The method is based on sequestration of magnesium ions in the form of a precipitate which renders a magnesium dependent enzyme inactive until the appropriate time in the reaction when a certain temperature is reached and the magnesium ions are released from the precipitate. Also provided are kits comprising reagents and instructions for amplifying a target nucleic acid, for DNA digestion and ligation, and for reverse transcription of RNA into cDNA. Furthermore, the kits and reagents of the present invention can be utilized in other reactions requiring magnesium dependent enzymes.

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US02/24533

A. CLASSIFICATION OF SUBJECT MATTER
IPC(7) : C12Q 1/68; C12P 19/34; C07H 21/04
US CL : 435/6, 91.1, 91.2, 91.21, 91.51; 536/23.1, 23.5, 24.33
According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
U.S. : 435/6, 91.1, 91.2, 91.21, 91.51; 536/23.1, 23.5, 24.33

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)
Please See Continuation Sheet

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	US RE 33,487 (MARKS) 11 December 1990 (11.12.1990), columns 8-9.	45-50, 63
X	US 5,565,339 A (BLOCH et al) 15 October 1996 (15.10.1996), see whole document, especially columns 30-32.	45-63
A	WO 96/00301 A1 (THE UNIVERSITY OF HOUSTON) 4 January 1996 (04.01.199), see whole document, especially, pages 4-22.	1-63
A	US 5,599,660 A (RAMANUJAM et al) 4 February 1997 (04.02.1997). see whole document, especially columns 2-13.	1-63
A	US 6,214,557 B1 (BARNES et al) 10 April 2001 (10.04.2001), see whole document, especially columns 1-20.	1-63

☐ Further documents are listed in the continuation of Box C. ☐ See patent family annex.

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Date of the actual completion of the international search	Date of mailing of the international search report
26 February 2003 (26.02.2003)	26 MAR 2003
Name and mailing address of the ISA/US Commissioner of Patents and Trademarks Box PCT Washington, D.C. 20231 Facsimile No. (703)305-3230	Authorized officer Felicia D. Roberts for Alexander H. Spiegler Telephone No. (703) 308-0196

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INTERNATIONAL SEARCH REPORT

PCT/US02/24533

Continuation of B. FIELDS SEARCHED Item 3:
Databases: US PAT, PG PUB, EPO, JPO, Derwent, Medline, Biosis, CaPlus, Embase, Biotechds

Terms: magnesium, phosphate, precipitate, temperature, enzyme, ligase, endonuclease, polymerase

Form PCT/ISA/210 (second sheet) (July 1998)

(12) INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(19) World Intellectual Property Organization
International Bureau

PCT

(43) International Publication Date
30 August 2007 (30.08.2007)

(10) International Publication Number
WO 2007/096182 A1

(51) International Patent Classification:
C12Q 1/68 (2006.01) *C07K 7/06* (2006.01)
C07K 5/113 (2006.01) *C07K 7/08* (2006.01)

(21) International Application Number: PCT/EP2007/001585

(22) International Filing Date: 23 February 2007 (23.02.2007)

(25) Filing Language: English

(26) Publication Language: English

(30) Priority Data:
06003903.9 27 February 2006 (27.02.2006) EP

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(81) Designated States *(unless otherwise indicated, for every kind of national protection available)*: AE, AG, AL, AM, AT, AU, AZ, BA, BB, BG, BR, BW, BY, BZ, CA, CH, CN, CO, CR, CU, CZ, DE, DK, DM, DZ, EC, EE, EG, ES, FI, GB, GD, GE, GH, GM, GT, HN, HR, HU, ID, IL, IN, IS, JP, KE, KG, KM, KN, KP, KR, KZ, LA, LC, LK, LR, LS, LT, LU, LV, LY, MA, MD, MG, MK, MN, MW, MX, MY, MZ, NA, NG, NI, NO, NZ, OM, PG, PH, PL, PT, RO, RS, RU, SC, SD, SE, SG, SK, SL, SM, SV, SY, TJ, TM, TN, TR, TT, TZ, UA, UG, US, UZ, VC, VN, ZA, ZM, ZW.

(84) Designated States *(unless otherwise indicated, for every kind of regional protection available)*: ARIPO (BW, GH, GM, KE, LS, MW, MZ, NA, SD, SL, SZ, TZ, UG, ZM, ZW), Eurasian (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European (AT, BE, BG, CH, CY, CZ, DE, DK, EE, ES, FI, FR, GB, GR, HU, IE, IS, IT, LT, LU, LV, MC, NL, PL, PT, RO, SE, SI, SK, TR), OAPI (BF, BJ, CF, CG, CI, CM, GA, GN, GQ, GW, ML, MR, NE, SN, TD, TG).

Declaration under Rule 4.17:
— *of inventorship (Rule 4.17(iv))*

Published:
— *with international search report*
— *with amended claims*
— *under Rule 91.1(f), with a request for rectification*

For two-letter codes and other abbreviations, refer to the "Guidance Notes on Codes and Abbreviations" appearing at the beginning of each regular issue of the PCT Gazette.

WO 2007/096182 A1

(54) Title: PCR HOT START BY MAGNESIUM SEQUESTRATION

(57) Abstract: The present invention is directed to Synthetic peptide having a length of not more than 30 amino acids comprising a divalent cation binding site. Such a peptide according to the present invention is part of a composition for nucleic acid amplification and provides for a so called hot start effect.

PCR Hot Start by Magnesium sequestration

Technical field

The present invention relates to the technical field of amplification of nucleic acids by means of performing the polymerase chain reaction process (PCR). More precisely, the present invention provides a new hot start alternative for performing PCR, which prevents unspecific priming events and the generation of false amplification products.

Prior Art Background

A major problem with nucleic acid amplification and more especially with PCR is the generation of unspecific amplification products. In many cases, this is due to an unspecific oligonucleotide priming and subsequent primer extension event prior to the actual thermocycling procedure itself, since thermostable DNA polymerases are also moderately active at ambient temperature. For example, amplification products due to eventually by chance occurring primer dimerisation and subsequent extension are observed frequently. In order to overcome this problem, it is well known in the art to perform a so called "hot start" PCR, wherein one component essential for the amplification reaction is either separated from the reaction mixture or kept in an inactive state until the temperature of the reaction mixture is being raised for the first time. Since the polymerase cannot function under these conditions, there is no primer elongation during the period when the primers can bind none specifically. In order to achieve this effect, several methods have been applied:

a) Physical separation of the DNA polymerase

The physical separation can be obtained for example by a barrier of solid wax, which separates the compartment containing the DNA polymerase from the compartment containing the bulk of the other reagents. During the first heating step the wax is then melting automatically and the fluid compartments are mixed (Chou, Q., et al., Nucleic Acids Res 20 (1992) 1717-23, US 5,411,876). Alternatively, the DNA polymerase is affinity immobilized on a solid support prior to the amplification reaction and only released into the reaction mixture by a heat mediated release (Nilsson, J., et al., Biotechniques 22 (1997) 744-51). Both methods, however are time consuming and inconvenient to perform.

b) Chemical modification of DNA polymerase

For this type of hot start PCR, the DNA polymerase is reversibly inactivated as a result of a chemical modification. More precisely, heat labile blocking groups are introduced into the Taq DNA polymerase which renders the enzyme inactive at room temperature (US 5,773,258). These blocking groups are removed at high temperature during a pre-PCR step such that the enzyme is becoming activated. Such a heat labile modification, for example can be obtained by coupling Citraconic Anhydride or Aconitric Anhydride to the Lysine residues of the enzyme (US 5,677,152). Enzymes carrying such modifications are meanwhile commercially availabile as Amplitaq Gold (Moretti, T., et al., Biotechniques 25 (1998) 716-22) or FastStart DNA polymerase (Roche Molecular Biochemicals). However, the introduction of blocking groups is a chemical reaction which arbitrarily occurs on all sterically available Lysine residues of the enzyme. Therefore, the reproducibility and quality of chemically modified enzyme preparations may vary and can hardly be controlled.

c) Recombinant modification of DNA polymerase

Cold sensitive mutants of Taq Polymerase have been prepared by means of genetic engineering. These mutants differ from the wildtype enzyme in that they lack the N-terminus (US 6,241,557). In contrast to native or wild type recombinant Taq Polymerase, these mutants are completely inactive below 35° C und thus may be used in some cases for performing a hot start PCR. However, the N-terminal truncated cold sensitive mutant form requires low salt buffer conditions, has a lower processivity as compared to the wild type enzyme and thus can only be used for the amplification of short target nucleic acids. Moreover, since the truncated form lacks 5'-3' exonuclease activity, it can not be used for real time PCR experiments based on the TaqMan detection format.

d) DNA polymerase inhibition by nucleic acid additives

Extension of non-specifically annealed primers has been shown to be inhibited by the addition of short double stranded DNA fragments (Kainz, P., et al., Biotechniques 28 (2000) 278-82). In this case, primer extension is inhibited at temperatures below the melting point of the short double stranded DNA fragment, but independent from the sequence of the competitor DNA itself. However, it is not known, to which extent the

excess of competitor DNA influences the yield of the nucleic acid amplification reaction.

Alternatively, oligonucleotide Aptamers with a specific sequence resulting in a defined secondary structure may be used. Such Aptamers have been selected using the SELEX Technology for a very high affinity to the DNA polymerase (US 5,693,502, Lin, Y., and Jayasena, S. D., J Mol Biol 271 (1997) 100-11). The presence of such Aptamers within the amplification mixture prior to the actual thermocycling process itself again results in a high affinity binding to the DNA polymerase and consequently a heat labile inhibition of its activity (US 6,020,130). Due to the selection process, however, all so far available Aptamers can only be used in combination with one particular species of DNA polymerase.

e) Taq DNA antibodies

An alternative approach to achieve heat labile inhibition of Taq DNA polymerase is the addition of monoclonal antibodies raised against the purified enzyme (Kellogg, D. E., et al., Biotechniques 16 (1994) 1134-7; Sharkey, D. J., et al., Biotechnology (N Y) 12 (1994) 506-9). Like the oligonucleotide Aptamers, the antibody binds to Taq DNA polymerase with high affinity at ambient temperatures in an inhibitory manner (US 5,338,671). The complex is resolved in a preheating step prior to the thermocycling process itself. This leads to a substantial time consuming prolongation of the amplification as a whole, especially if protocols for rapid thermocycling are applied (WO 97/46706).

US 5,985,619 discloses a specific embodiment for performing PCR using a hot start antibody, wherein besides Taq polymerase, e. g. Exonuclease III from E. coli is added as a supplement to the amplification mixture in order to digest unspecific primer dimer intermediates. As disclosed above, Exonuclease III recognizes double-stranded DNA as a substrate, like, for example, target/primer- or target/primer extension product hybrids. Digestion is taking place by means of cleavage of the phosphodiester bond at the 5' end of the 3' terminal deoxynucleotide residue. Since this type of exonuclease is active at ambient temperatures, all unspecifically annealed primers and primer extension products therefore are digested. This results in some embodiments in an even enhanced specificity of the amplification reaction. Yet, digestion of the unspecific primers dependent on the duration of the preincubation time may lead to a substantial

and uncontrolled decrease in primer concentration, which in turn may affect the amplification reaction itself.

f) Usage of modified primers alone or in combination with exonucleases

EP 0 799 888 and GB 2293238 disclose an addition of 3' blocked oligonucleotides to PCR reactions. Due to the 3' block, these oligonucleotides can not act as primers. The blocked oligonucleotides are designed to compete/interact with the PCR primers which results in reduction of non-specific products.

Another alternative is the use of phosphorothioate oligonucleotide primers in combination with an exonuclease III in the PCR reaction mixes (EP 0 744 470). In this case, a 3′ exonuclease, which usually accepts double stranded as well as single stranded DNA substrates, degrades duplex artefacts such as primer dimers as well as carry over amplicons, while leaving the single stranded amplification primers undegraded. Similarly, the usage of primers with a basic modified 3' ends and template dependent removal by E.coli Endonuclease IV has been suggested (US 5,792,607).

A particular embodiment of the general idea is found in EP 1 275 735. Its specification discloses a composition for performing a nucleic acid amplification reaction comprising (i) a thermostable DNA-Polymerase, (ii) a thermostable 3'-5' Exonuclease, and (iii) at least one primer for nucleic acid amplification with a modified 3' terminal residue which is not elongated by said thermostable DNA-Polymerase as well as methods for performing a PCR reaction using this composition. Furthermore, the method is directed to kits comprising such a composition.

However, it is major drawback of the disclosed alternatives that for each PCR reaction, modified primers are required, which lead to increased requirements regarding increase the cost for each individual assay.

g) other PCR additives

Other organic additives known in the art like DMSO, betaines, and formamides (WO 99/46400; Hengen, P. N., Trends Biochem Sci 22 (1997) 225-6; Chakrabarti, R., and Schutt, C. E., Nucleic Acids Res 29 (2001) 2377-81) result in an improvement of amplification of GC rich sequences, rather than prevention of primer dimer formation. Similarly, heparin may stimulate in vitro run-on transcription presumably by removal

of proteins like histones in order to make chromosomal DNA accessible (Hildebrand, C. E., et al., Biochimica et Biophysica Acta 477 (1977) 295-311).

It is also known that addition of single strand binding protein (US 5,449,603) or tRNA, (Sturzenbaum, S. R., Biotechniques 27 (1999) 50-2) results in non-covalent association of these additives to the primers. This association is disrupted when heating during PCR. It was also found that addition of DNA helicases prevent random annealing of primers (Kaboev, O. K., et al., Bioorg Khim 25 (1999) 398-400). Furthermore, poly-glutamate (WO 00/68411) in several cases may be used in order to inhibit polymerase activity at low temperatures.

Moreover, it is known that polyanionic polymerase inhibitors may control the activity of thermostable DNA polymerases dependent on the applied incubation temperature. US 6,667,165 discloses a hot start embodiment, characterized in that inactive polymerase-inhibitor complexes are formed at temperatures below 40° C. Between 40° C and 55° C, the inhibitor competes with the template DNA for binding to the Taq Polymerase, whereas at temperatures above 55° C, the inhibitor is displaced from the polymerase active site. Yet, the inhibitor tends to reduce the obtainable product yield, when primers with lower annealing temperatures are used.

h) Magnesium sequestration

Since thermostable polymerases are known for a long time to be active only in presence of Mg2+ cations, a sequestration of magnesium prior to the start of the thermocycling protocol has been attempted in order to avoid mispriming and unspecifying primer extension. As disclosed in US 6,403,341, Mg2+ may be present in form of a precipitate and thus unavailable at the beginning of the amplification reaction. Upon temperature increase during the first round of thermocycling, the precipitate dissolves and Mg2+ becomes fully available within the first 3 cycles. Such a solution has been shown to be fairly applicable and capable of providing good hot start results. On the other hand, such a solution does not allow the preparation of mastermixes containing all reagents except primer and target nucleic acid which are necessary to perform a nucleic acid amplification reaction. As a consequence, inter-assay data reproducibility and data comparisons are complicated.

In view of the outlined prior art it was an object of the invention to provide an improved alternative composition and method for hot start PCR, which allows for an inhibition of unspecific priming and primer extension not only prior to the amplification process itself but also during the thermocycling process. More precisely, it was an object of the invention to provide an alternative composition and method for hot start PCR, where no extension of unspecifically annealed primers can take place.

Brief description of the invention

Most thermostable polymerases capable of catalyzing a polymerase chain reaction are dependent on the presence of a divalent cation, usually Mg2+. The present invention is based on the principle of generating a hot start effect by adding a divalent cation binding compound to a polymerase chain reaction mixture, which binds divalent cations in a temperature dependent manner.

Thus, in a first aspect, the present invention is directed to a synthetic peptide having a length of not more than 30 amino acids comprising a divalent cation binding site.

Preferably, said synthetic peptide according to the present invention binds said divalent cation with an affinity constant between 0.01 mM and 10 000 μM.

Since most thermostable polymerases are Mg2+ dependent, said divalent cation binding site is preferably a motive which binds Mg2+.

In a specific embodiment, the synthetic peptide according to the present invention comprises the amino acid sequence motive X1X2X3 at least once, wherein

X1 is a negatively charged amino acid, preferentially Aspartic acid
X2 is either Glycine or an aliphatic amino acid
X3 is a negatively charged amino acid

In a second aspect, the present invention is directed to a composition comprising

- a thermostable DNA Polymerase
- at least one sort of a divalent cation, preferably Mg2+
- deoxynucleotides
- a buffer

- a synthetic peptide a having a length of not more than 30 amino acids comprising a divalent cation binding site as disclosed above

Preferably, said composition according to the present invention further comprises at least one nucleic acid compound such as a primer and/or a target nucleic acid that shall become amplified.

In a third aspect, the present invention is directed to a kit comprising

- a peptide as disclosed above, and
- a thermostable DNA Polymerase

In a forth aspect the present invention is directed to a method for amplification of a specific target nucleic acid comprising the steps of

- providing a sample suspected to contain said target nucleic acid
- adding a composition as disclosed above, and
- performing a Polymerase Chain Reaction

In a specific embodiment, said Polymerase Chain Reaction is monitored in real time.

In a further specific embodiment, the amplification product generated by said amplification is subjected to a melting curve analysis.

Brief description of figures

Figure 1

Figure 1 shows the effect of magnesium binding peptide with the sequence DIETDIET as disclosed in example 1. The primer dimer product formation (⇒) is suppressed when the peptide is present in the PCR reaction mixture without influence on specific product formation (⟶).

Lanes	
1:	molecular weight marker VI from Roche Applied Science
2, 7:	50 ng of template DNA
3, 8;	25 ng of template DNA
4, 9:	10 ng of template DNA
5, 10:	5 ng of template DNA
6, 11:	1 ng of template DNA

Products of lanes 2 to 6 were amplified in the absence of peptide, products of lanes 7 to 11 in the presence of 2 mM of the peptide with the sequence H-DIETDIET-NH2.

The primer dimer product formation (⟹) is suppressed when the peptide is present in the PCR reaction mixture without influence on specific product formation(⟶).

Figure 2

Figure 2 shows the effect of magnesium binding peptide of the sequence H-FDGDFDGD-NH2. Primer dimer product formation (⟹) is reduced when the peptide is present in the PCR reaction, in parallel an increase of the specific product formation (⟶) is observed.

Lane 1: molecular weight marker VI from Roche Applied Science
2: 25 ng of target DNA, no peptide
3: 25 ng of target DNA, 3 mM of peptide
4: 10 ng of target DNA, no peptide
5: 10 ng of target DNA, 3 mM of peptide

Figure 3

Figure 3A: RT-PCR with 102 to 106 copies of G6PDH RNA in the absence of magnesium binding peptide.

Figure 3b: Melting curve analysis of RT-PCR with 102 to 106 copies of G6PDH RNA in the absence of magnesium binding peptide.

Figure 3c: RT-PCR with 102 to 106 copies of G6PDH RNA in the presence of 1 mM of magnesium binding peptide.

Figure 3d: Melting curve analysis of RT-PCR with 102 to 106 copies of G6PDH RNA in the presence of 1 mM of magnesium binding peptide.

Figure 4

Products of lanes 2 to 6 were amplified in the absence of the peptide H-DIETDIETDIET-NH2, products of lanes 7 to 11 in the presence of 2.0 mM of the peptide with the sequence H-DIETDIETDIET-NH2. The primer dimer product formation (⟹) is suppressed when the peptide is present in the PCR reaction mixture without influence on specific product formation(⟶).

Lanes	1, 12:	molecular weight marker V from Roche Applied Science
	2, 7:	50 ng of template DNA
	3, 8:	25 ng of template DNA
	4, 9:	10 ng of template DNA
	5, 10:	5 ng of template DNA
	6, 11:	1 ng of template DNA

Detailed description of the present invention

Most thermostable polymerases capable of catalyzing a polymerase chain reaction are dependent on the presence of a divalent cation, usually Mg2+. The present invention is based on the principle of generating a hot start effect by adding a divalent cation binding compound to a polymerase chain reaction mixture, which binds divalent cations in a temperature dependent manner.

Thus, in a first aspect, the present invention is directed to a synthetic peptide having a length of not more than 30 amino acids comprising a divalent cation binding site. Smaller synthetic peptides having a length of less than 17 amino acids may also be used. Since a binding site for divalent cations requires at least 3-4 amino acid residues (see below), the lower size limit is a peptide consisting of 3-4 amino acids representing a monomer of a divalent cation binding site. It is also within the scope of the present invention, if said synthetic peptides contain more than one divalent cation binding site motives, i.e. if they contain said motives twice, three times or four times. However, said synthetic peptides according to the present invention in addition to amino acids representing a divalent cation binding sequence motive may contain further amino acids, which are not part of such a motive, but may contribute to other features of said peptide. For example, additional amino acid residues may increase the solubility of said peptides in different solvents.

In the context of the present invention, the term „synthetic peptide“ is defined as a chain of amino acids connected by amide bonds, which has been chemically synthesized by means of condensation. Explicitly excluded, however, are peptides or peptide fragments which have been obtained from living organisms by means of isolation and (optional) fragmentation.

Such synthetic peptides can be used according to methods which are generally well known in the art. The synthesis is based on an automated cycling reaction wherein

amino acids are connected to the nascent peptide chain in a condensation reaction by means of forming an amide bond. Reactive side chains of the coupled amino acids are covered by appropriately removable protective groups. A comprehensive overview of the state of the art is given in Fields, G.B., Noble, R.L., J. Peptide Protein Res. 35 (1990)
5 161-214. Such a chemical synthesis results in the generation of peptides having a typical H_2N-Terminus (usually referenced as "H") and a C-Terminus with a carboxy-amidate (usually referenced as "-NH_2").

The peptides according to the present invention are capable of binding divalent cations. The strength of binding predominantly depends on the nature of the divalent cation in
10 conjunction with the primary peptide sequence motive of the peptide. Preferably, said synthetic peptide according to the present invention binds said divalent cation with an affinity constant between 0.01 mM and 10 000 µM at neutral pH. Compounds with stronger affinity rates such as EDTA have been shown to result in detrimental effects when added to an amplification reaction, whereas on he other hand, a minimal affinity
15 rate is required in order to generate a measurable positive effect when such a peptide compound is added to a nucleic acid amplification reaction. More preferably, said affinity constant has a value between 0.1 mM and 1000 mM. Most preferably, said affinity constant has a value between 1 mM and 100 mM.

Since most thermostable polymerases are Mg2+ dependent, said divalent cation
20 binding site is preferably a motive which binds Mg2+.

In the section below, the following classification of amino acids is applied.

No subclass:

Glycine (Gly) G
Proline (Pro) P

25 Non polar, aliphatic:

Alanine (Ala), A
Valine (Val), V
Leucine (Leu), L
Isoleucine (Ile) I

Aromatic:

Phenylalanine (Phe), F
Tyrosine (Tyr), Y
Tryptophane (Trp) W

5 Polar, uncharged:

Serine (Ser), S
Threonine (Thr), T
Cysteine (Cys), C
Methionine (Met), M
10 Asparagine (Asn), N
Glutamine (Gln), O

Positively charged:

Lysine (Lys), K
Arginine (Ag), R
15 Histidine (His) H

Negatively charged:

Aspartic acid (Asp), D
Glutamic acid (Glu) E

Preferably, the synthetic peptide according to the present invention comprises the
20 amino acid sequence motive X1X2X3 at least once, wherein

X1 is a negatively charged amino acid, preferentially Aspartic acid
X2 is either Glycine or an aliphatic amino acid
X3 is a negatively charged amino acid.

In one embodiment, X3 is a Glutamic acid.

25 If this is the case, X2 is preferably Isoleucine. Also preferably, c-terminal adjacent to X3 there is an X4 which is a polar, uncharged amino acid such as Threonine.

Most preferably, X2 is Isoleucine AND c-terminal adjacent to X3 there is an X4 which

is a polar uncharged amino acid such as Threonine.

In another embodiment, X3 is an Aspartic acid.

If this is he case, X2 is preferably Glycine. Also preferably, N-terminal adjacent to X1 there is an X0 which is an aromatic amino acid such as Phenylalanine.

5 Most preferably, X2 is Isoleucine AND N-terminal adjacent to X1 there is an X0 which is an aromatic amino acid such as Phenylalanine.

In a second aspect, the present invention is directed to a composition comprising

- a thermostable DNA Polymerase
- at least one sort of a divalent cation, preferably Mg2+
- 10 deoxynucleotides
- a buffer
- a synthetic peptide having a length of not more than 30 amino acids comprising a divalent cation binding site as disclosed above.

Such a composition according to the present invention is being used to perform a
15 nucleic acid amplification reaction in the form of a Polymerase Chain Reaction (PCR).

As thermostable polymerases, a great variety of enzymes may be used. Preferably, said thermostable DNA polymerase is selected from a group consisting of Aeropyrum pernix, Archaeoglobus fulgidus, Desulfurococcus sp. Tok., Methanobacterium thermoautotrophicum, Methanococcus sp. (e.g. jannaschii, voltae), Methanothermus
20 fervidus., Pyrococcus species (furiosus, species GB- D, woesii, abysii, horikoshii, KOD, Deep Vent, Proofstart), Pyrodictium abyssii, Pyrodictium occultum, Sulfolobus sp. (e.g. acidocaldarius, solfataricus), Thermococcus species (zilligii, barossii, fumicolans, gorgonarius, JDF-3, kodakaraensis KODI, litoralis, species 9 degrees North-7, species JDF-3, gorgonarius, TY), Thermoplasma acidophilum, Thermosipho africanus,
25 Thermotoga sp. (e.g. maritima, neapolitana), Methanobacterium thermoautotrophicum, Thermus species (e.g. aquaticus, brockianus, filiformis, flavus, lacteus, rubens, ruber, thermophilus, ZO5 or Dynazyme). Also within the scope of the present invention are mutants, variants or derivatives thereof, chimeric or "fusion-polymerases" e.g. Phusion (Finnzymes or New England biolabs, Catalog No. F-530S)
30 or iProof (Biorad, Cat. No. 172-5300), Pfx Ultima (Invitrogen, Cat. No. 12355012) or

Herculase II Fusion (Stratagene, Cat. No. 600675). Furthermore, compositions according to the present invention may comprise blends of one or more of the polymerases mentioned above.

In one embodiment, the thermostable DNA Polymerase is a DNA dependent polymerase. In another embodiment, the thermostable DNA polymerase has additional reverse transcriptase activity and may be used for RT-PCR. One example for such enzyme is Thermus thermophilus (Roche Diagnostics cat. No: 11 480 014 001). Also within the scope of the present invention are blends of one or more of the polymerases compiled above with retroviral reverse transcriptases, e.g. polymerases from MMLV, AMV, AMLV, HIV, EIAV, RSV and mutants of these reverse transcriptases.

The synthetic peptide having a length of not more than 30 amino acids comprising a divalent cation binding site is present in a concentration which allows for a „hot start effect", once the composition is used to amplify a target nucleic acid. Usually, the peptide comprising the Mg2+ binding site may be present in a final concentration between 0.1 and 10 mM. Preferably, the peptide comprising the Mg2+ binding site is present between 0.5 and 5 mM. Highly preferred are concentrations between 1 and 3 mM.

The concentrations of the DNA polymerase, the deoxynucleotide and the other buffer components are present in standard amounts, the concentrations of which are well known in the art. The Mg2+ concentration may vary between 0.1 mM and 3 mM and is preferably adapted and experimentally optimized. However, since the concentration optimum usually depends on the actual primer sequences used, it can not be predicted theoretically.

In addition to the compositions disclosed above it is also within the scope of the present invention, if these compositions further comprise at least one nucleic acid compound. For example, such a composition may comprise at least one pair of amplification primers useful for performing a nucleic acid amplification reaction. A composition according to the present invention may also be a PCR reaction mixture, which additionally comprises an at least partially purified nucleic acid sample, from which a target nucleic acid sequence suspected to be present in said sample shall be amplified.

Furthermore, such a composition may comprise fluorescent compounds for detecting a respective amplification product in real time and respectively 2, 3, 4, or 5-6 differently labeled hybridization probes not limited to but being selected from a group consisting of FRET hybridization probes, TaqMan probes, Molecular Beacons and Single labeled probes, useful for methods of detection as they will be disclosed below. Alternatively, such a composition may contain a dsDNA binding fluorescent entity such as SybrGreen which emits fluorescence only when bound to double stranded DNA.

In a third aspect, the present invention is directed to a kit at least comprising

(i) a thermostable DNA Polymerase
(ii) a synthetic Mg2+ binding peptide as disclosed above, and

Preferably, such a kit comprises at least

(i) a thermostable DNA Polymerase
(ii) a synthetic Mg 2+ binding peptide as disclosed above, and
(iii) a MgCl2 stock solution in order to adjust the final Mg2+ concentration

Most preferably, such a kit comprises at least

(i) a thermostable DNA Polymerase
(ii) a synthetic Mg 2+ binding peptide as disclosed above
(iii) a MgCl2 stock solution in order to adjust the final Mg2+ concentration
(iv) a mixture of deoxynucleoside-tri-phosphates, and
(v) a buffer

In addition, such a kit according to the present invention may be a parameter specific kit comprising at least one pair of amplification primers. Such a kit may also comprise multiple pairs of amplification primers and preferably 2, 3, 4 or 5-pairs of amplification primers.

Furthermore, such a kit may comprise fluorescent compounds for detecting a respective amplification product in real time and respectively 2, 3, 4, or 5-6 differently labeled hybridization probes not limited to but being selected from a group consisting of FRET hybridization probes, TaqMan probes, Molecular Beacons and Single labeled

probes, useful for methods of detection as they will be disclosed below. Alternatively, such a kit may contain a dsDNA binding fluorescent entity which emits fluorescence only when bound to double stranded DNA. For example, such a kit may comprise SybrGreen.

5 In one exemplary embodiment, such a kit is specifically adapted to perform one-step RT-PCR and comprises a blend of Taq DNA Polymerase and a reverse transcriptase such as AMV reverse transcriptase. In a further exemplary particular embodiment, such a kit is specifically adapted to perform one-step real time RT-PCR and comprises a nucleic acid detecting entity such as SybrGreen or a fluorescently labeled nucleic acid
10 detection probe.

The different components may be stored each individually in different vessels. Alternatively, the different components may be stored all together in one storage vessel. Also alternatively, arbitrarily selected combinations of only a subset of components (i) to (v) may be stored together. In a preferred embodiment, components like enzyme (i)
15 or enzyme plus reaction buffer containing MgU_2, dNTPs (i, ii, iv, v) and the peptide are stored together.

In a forth aspect the present invention is directed to a method for amplification of a specific target nucleic acid comprising the steps of

- providing a sample suspected to contain said target nucleic acid
20 - adding a composition as disclosed above
- performing a nucleic acid amplification reaction

Methods for performing and optimizing nucleic acid amplification reactions are well known in the art. In particular, the most common method used in the art is the polymerase chain reaction as disclosed in detail in US 4,683,195, US 4,683,202 and
25 US 4,965,188.

In particular, the method according to the present invention comprises the steps of

a) providing a sample suspected to contain a target nucleic acid that shall be amplified

b) providing a synthetic peptide having a length of not more than 30 amino acids comprising a divalent cation binding site

c) providing DNA polymerase, dNTPs, a buffer and a Mg2+ salt

d) providing a pair of amplification primers designed to specifically amplify a predefined target nucleic acid.

Addition of all components mentioned can be done in arbitrary order. However, in order to achieve a hot start effect, i.e. in order to prevent a mispriming and subsequent primer extension at lower temperatures prior to the thermocycling amplification protocol itself, it is important that said synthetic peptide is being added to the reaction mixture, before the DNA polymerase, the dNTPs and the amplification primers are combined.

Although the mechanism how said synthetic peptides comprising a divalent cation binding site result in more specific amplification reactions is not known in detail, it is reasonable to assume that at lower temperatures, the peptide forms a complex with Mg2+ which itself is known to be a co-factor of DNA polymerases in primer extension reactions. Quantitative complex formation results in a decrease in the concentration of free Mg2+ with the consequence that the DNA Polymerase activity is inactivated due to a lack of availability of its cofactor. After initiation of the temperature cycling protocol, the thermo-labile complex between the synthetic peptide and the divalent cation is resolved, and Mg2+ is available again as a cofactor for Polymerase catalyzed primer extension reactions.

Preferably, the synthetic peptide added during nucleic acid amplification comprises the amino acid sequence motive X1X2X3 at least once, wherein

X1 is a negatively charged amino acid, preferentially Aspartic acid
X2 is either Glycine or an aliphatic amino acid
X3 is a negatively charged amino acid.

In one embodiment, X3 is a Glutamic acid.

If this is the case, X2 is preferably Isoleucine. Also preferably, C-terminal adjacent to X3 there is an X4 which is a polar, uncharged amino acid such as Threonine.

Most preferably, X2 is Isoleucine AND C-terminal adjacent to X3 there is an X4 which is a polar uncharged amino acid such as Threonine.

In another embodiment, X3 is an an Aspartic acid.

If this is the case, X2 is preferably Glycine. Also preferably, N-terminal adjacent to X1 there is an X0 which is an aromatic amino acid such as Phenylalanine.

Most preferably, X2 is Isoleucine AND N-terminal adjacent or X1 there is an X0 which is an aromatic amino acid such as Phenylalanine.

In a specific embodiment, said Polymerase Chain Reaction is monitored in real time. There are different detection formats for such a monitoring.

a) TaqMan format:

A single-stranded Hybridization Probe is labeled with two components. When the first component is excited with light of a suitable wavelength, the absorbed energy is transferred to the second component, the so-called quencher, according to the principle of fluorescence resonance energy transfer. During the annealing step of the PCR reaction, the hybridization probe binds to the target DNA and is degraded by the 5'-3' exonuclease activity of the Taq Polymerase during the subsequent elongation phase. As a result the excited fluorescent component and the quencher are spatially separated from one another and thus a fluorescence emission of the first component can be measured. TaqMan probe assays are disclosed in detail in US 5,210,015, US 5,538,848, and US 5,487,972. TaqMan hybridization probes and reagent mixtures are disclosed in US 5,804,375.

b) Releasing formats:

Moreover, two other formats restricted to allele specific detection have been disclosed recently which are based on the principle of specific detection of a release of a labeled 3' terminal nucleotide due to a match or mismatch situation regarding its binding to the target nucleic acid. US 6,391,551 discloses a method, characterized in that the 3' terminal nucleotide of a hybridization probe is released by a depolymerizing enzyme in case a perfect match between target sequence and probe has occurred. Similarly, EP 0 930 370 suggests to use a primer labeled with a reporter and a quencher moiety,

characterized in that a 3'-5' proofreading activity removes one moiety in case no perfect match between primer and amplification target has occurred.

c) Molecular Beacons:

These hybridization probes are also labeled with a first component and with a quencher, the labels preferably being located at both ends of the probe. As a result of the secondary structure of the probe, both components are in spatial vicinity in solution. After hybridization to the target nucleic acids both components are separated from one another such that after excitation with light of a suitable wavelength the fluorescence emission of the first component can be measured (US 5,118,801).

d) FRET hybridization probes:

The FRET Hybridization Probe test format is especially useful for all kinds of homogenous hybridization assays (Matthews, J.A., and Kricka, L.J., Analytical Biochemistry 169 (1988) 1-25). It is characterized by two single-stranded hybridization probes which are used simultaneously and are complementary to adjacent sites of the same strand of the amplified target nucleic acid. Both probes are labeled with different fluorescent components. When excited with light of a suitable wavelength, a first component transfers the absorbed energy to the second component according to the principle of fluorescence resonance energy transfer such that a fluorescence emission of the second component can be measured when both hybridization probes bind to adjacent positions of the target molecule to be detected. Alternatively to monitoring the increase in fluorescence of the FRET acceptor component, it is also possible to monitor fluorescence decrease of the FRET donor component as a quantitative measurement of hybridization event.

In particular, the FRET Hybridization Probe format may be used in real time PCR, in order to detect the amplified target DNA. Among all detection formats known in the art of real time PCR, the FRET-Hybridization Probe format has been proven to be highly sensitive, exact and reliable (WO 97/46707; WO 97/46712; WO 97/46714). As an alternative to the usage of two FRET hybridization probes, it is also possible to use a fluorescent-labeled primer and only one labeled oligonucleotide probe (Bernard, P.S., et al., Analytical Biochemistry 255 (1998) 101-107). In this regard, it may be chosen

arbitrarily, whether the primer is labeled with the FRET donor or the FRET acceptor compound.

e) Single Label Probe (SLP) format:

This detection format consists of a single oligonucleotide labeled with a single fluorescent dye at either the 5‘- or 3‘-end (WO 02/14555). Two different designs can be used for oligo labeling: G-Quenching Probes and Nitroindole-Dequenching probes. In the G-Quenching embodiment, the fluorescent dye is attached to a C at oligo 5‘- or 3‘-end. Fluorescence decreases significantly when the probe is hybridized to the target, in case two G's are located on the target strand opposite to C and in position 1 aside of complementary oligonucleotide probe. In the Nitroindole Dequenching embodiment, the fluorescent dye is attached to Nitroindole at the 5‘- or 3‘-end of the oligonucleotide. Nitroindole somehow decreases the fluorescent signaling of the free probe. Fluorescence increases when the probe is hybridized to the target DNA due to a dequenching effect.

f) SybrGreen format:

It is also within the scope of the invention, if real time PCR is performed in the presence of an additive according to the invention in case the amplification product is detected using a double stranded nucleic acid binding moiety. For example, the respective amplification product can also be detected according to the invention by a fluorescent DNA binding dye which emits a corresponding fluorescence signal upon interaction with the double-stranded nucleic acid after excitation with light of a suitable wavelength. The dyes SybrGreenI and SybrGold (Molecular Probes) have proven to be particularly suitable for this application. Intercalating dyes can alternatively be used. However, for this format, in order to discriminate the different amplification products, it is necessary to perform a respective melting curve analysis (US 6,174,670).

A further aspect of the present invention is directed to methods characterized in that a synthetic peptide having a length of not more than 30 amino acids comprising a divalent cation binding site as disclosed above is used for real time PCR and subsequent melting curve analysis.

Due to the fact that real time amplicon detection with SybrGreen format can not discriminate between specific products and amplification artefacts such as primer/dimers, a subsequent melting curve analysis is usually performed. After completion of the PCR-reaction, the temperature of the sample is constitutively increased, and fluorescence is detected as long as SybrGreen is bound to the double stranded DNA present in the samples. Upon dissociation of the double stranded DNA the signal decreases immediately. This decrease is monitored with an appropriate fluorescence versus temperature-time plot such that a first derivative value can be determined, at which the maximum of fluorescence decrease is observed. Since primer/dimer double stranded DNAs are usually short, dissociation into single stranded DNA occurs at lower temperatures as compared to the dissociation of the double stranded specific amplification product.

If during such a melting curve analysis, a synthetic peptide with a divalent cation binding site is present within the sample, much sharper curves are obtained in many cases, when the first derivative of a respective fluorescence versus temperature-time plot is determined.

Besides PCR and real time PCR, FRET hybridization probes are used for melting curve analysis. In such an assay, the target nucleic acid is amplified first in a typical PCR reaction with suitable amplification primers. The hybridization probes may already be present during the amplification reaction or added subsequently. After completion of the PCR-reaction, the temperature of the sample is constitutively increased, and fluorescence is detected as long as the hybridization probe was bound to the target DNA. At melting temperature, the hybridization probes are released from their target, and the fluorescent signal is decreasing immediately down to the background level. This decrease is monitored with an appropriate fluorescence versus temperature-time plot such that a first derivative value can be determined, at which the maximum of fluorescence decrease is observed.

If during such a melting curve analysis, Synthetic peptide having a length of not more than 30 amino acids comprising a divalent cation binding site is present within the sample, much sharper curves are obtained, when the first derivative of a respective fluorescence versus temperature-time plot is determined. Similar effects can be observed, if either molecular beacons or single labeled probes are used as detecting entities for melting curve analysis.

The following examples, sequence listing and figures are provided to aid the understanding of the present invention, the true scope of which is set forth in the appended claims. It is understood that modifications can be made in the procedures set forth without departing from the spirit of the invention.

5 Examples

Example 1:

A peptide with the sequence H-DIETDIET-NH2 was synthesized, HPLC purified, lyophilized and dissolved in 30 mM Tris-HCl, pH 8.5. PCR reactions were performed in 50 µl volume containing 50 ng, 25 ng, 10 ng, 5 ng or 1 ng of human genomic DNA,
10 2.5 units Taq polymerase (Roche Applied Science Cat. No. 11146165001), 0.4 mM forward primer (aga cag tac agc cag cct ca) (SEQ ID No: 1), 0.4 mM reverse primer (gac ttc aaa ttt ctg ctc ctc) (SEQ ID NO: 2), 0.2 mM dATP, dCTP, dGTP and dCTP, Taq PCR reaction buffer (Roche Applied Science Cat. No. 11146165001), with and without H-DIETDIET-NH2-Peptide. The PCR was performed as follows: 2 min at
15 94° C, 35 cycles with 10 sec at 94° C, 30 sec at 60° C and 30 sec at 72° C and a final elongation step over 7 min at 72° C. The PCR products were analyzed by agarose gel electrophoresis.

As it can be seen in fig. 1, addition of 2.0 mM of the disclosed peptide results in a significant decrease of generation of primer/dimer amplification products.

20 Example 2:

Analysis of a peptide with the sequence H-FDGDFDGD-NH2. PCR reactions were performed in 50 µl volume containing 25 ng or, 10 ng of human genomic DNA, 2.5 units Taq polymerase (Roche Applied Science Cat. No. 11146165001), 0.4 mM forward primer (aga cag tac agc cag cct ca) (SEQ ID NO: 1), 0.4 mM reverse primer (agt atg ccc
25 ccg cac agg a) (SEQ ID NO: 3), 0.2 mM dATP, dCTP, dGTP and dCTP, Taq PCR reaction buffer (Roche Applied Science Cat. No. 11146165001), with and without H-FDGDFDGD -NH2-Peptide. The PCR cycling conditions were as follows: 2 min at 94° C, 35 cycles with 10 sec at 94° C, 30 sec at 60° C and 30 sec at 72° C and a final elongation step over 7 min at 72° C. The PCR products were analyzed by agarose gel
30 electrophoresis.

As it can be seen in fig. 2, addition of 3 mM H-FDGDFDGD-NH2 results in decreased primer/dimer product formation. In parallel an increase of the specific product formation is observed.

Example 3:

5 The peptide with the sequence H-DIETDIET-NH2 was analyzed in real time RT-PCR. PCR reactions were performed in 20 µl volume containing 10^2, 10^3, 10^4, 10^5 and 10^6 copies of RNA standard available in the LightCycler h-G6PDH Housekeeping gene kit from Roche Applied Science (Cat. No.: 3261883), 2.4 units Transcriptor and 1.6 units FastStart polymerase, reaction buffer from the LightCycler RNA Amplification Kit
10 SYBR Green (Roche Applied Science Cat. No. 12 015 137 001), 0.5 mM forward primer (ggg tgc atc ggg tga cct g) (SEQ ID NO: 4), 0.5 mM reverse primer (agc cac tgt gag gcg gga) (SEQ ID NO: 5), with and without 1 mM H-DIETDIET-NH2-peptide. The PCR was performed in a LightCycler 2.0 as follows: 10 min at 55° C, 10 min at 95° C, 45 cycles with 10 sec at 95° C, 10 sec at 55 ° C and 13 sec at 72° C.

15 The result of this example is shown in Figure 3. It illustrates that a magnesium binding peptide reduces primer-dimer formation in RT-PCR. The amplification curves (Fig 3a) show that in the absence of peptide unspecific product is formed. PCR products amplified from 102, 103 and 104 copies of target are detected at similar cp-values. The amplification curves may be derived from several products formed. In the melting
20 curve analysis depicted in Figure 3b two melting profiles are detectable with 103 and 102 copies of target RNA. With these dilutions of target the melting curves show two products.

Figure 3c shows amplification curves in the presence of 1 mM magnesium binding peptide. The amplification products of the target dilutions are detected at increasing
25 crossing points, the amplification curves are well separated. An increase of specificity compared to the experiment without magnesium binding peptide is also observed in the melting curve analysis of Figure 3d. One melting curve with the Tm of the specific product is exclusively detected the target dilutions from 106 to 103. In the sample with 102 copies of target the main product is the specific product, very little primer-dimer is
30 observed.

Example 4:

A peptide with the sequence H-DIETDIETDIET-NH2 (SEQ ID NO: 8) was synthesized, HPLC purified, lyophilized and dissolved in 30 mM Tris-HCl, pH 8.5. PCR reactions were performed in 50 µl volume containing 50 ng, 25 ng, 10 ng, 5 ng or 1 ng of human genomic DNA, 2.5 units Taq polymerase (Roche Applied Science Cat. No. 11146165001), 0.4 mM forward primer (cac ccc gtg ctg ctg acc ga) (SEQ ID NO: 6), 0.4 mM reverse primer (agg gag gcg gcc acc aga ag) (SEQ ID NO: 7), 0.2 mM dATP, dCTP, dGTP and dCTP, Taq PCR reaction buffer (Roche Applied Science Cat. No. 11146165001), with and without H-DIETDIETDIET-NH2 peptide in amounts as indicated in Figure 4. The PCR was performed as follows: 2 min at 94° C, 35 cycles with 10 sec at 94° C, 30 sec at 65° C and 15 sec at 72° C and a final elongation step over 7 min at 72° C. The PCR products were analyzed by agarose gel electrophoresis.

Figure 4 shows the effect of magnesium binding peptide with the sequence DIETDIETDIET. The primer dimer product formation (⟹) is suppressed when the peptide is present in the PCR reaction mixture without influence on specific product formation(⟶).

Example 5:

A peptide with the sequence H-DIET-NH2 was synthesized, HPLC purified, lyophilized and dissolved in 30 mM Tris-HCl, pH 8.5. PCR reactions were performed in 50 µl volume containing 50 ng, 25 ng, 10 ng, 5 ng or 1 ng of human genomic DNA, 2.5 units Taq polymerase (Roche Applied Science Cat. No. 11146165001), 0.4 mM forward primer (aga cag tac agc cag cct ca) (SEQ ID NO: 1), 0.4 mM reverse primer (gac ttc aaa ttt ctg ctc ctc) (SEQ ID NO: 2), 0.2 mM dATP, dCTP, dGTP and dCTP, Taq PCR reaction buffer (Roche Applied Science Cat. No. 11146165001), with and without H-DIET-NH2-Peptide. The PCR was performed as follows: 2 min at 94° C, 35 cycles with 10 sec at 94° C, 30 sec at 60° C and 30 sec at 72° C and a final elongation step over 7 min at 72° C. The PCR products were analyzed by agarose gel electrophoresis. A decrease in primer/dimer formation was observed in the presence of at least 3 mM H-DIET-NH2.

Patent Claims

1. Synthetic peptide having a length of not more than 30 amino acids comprising a divalent cation binding site

2. Peptide according to claim 1, which binds said divalent cation with an affinity constant between 0.01mM and 10 000 μM
5
3. Peptide according to claims 1-2, which binds Mg2+

4. Peptide according to claim 3, comprising the peptide sequence X1X2X3 at least once
wherein
10 X1 is a negatively charged amino acid, preferentially aspartic acid
X2 is either Glycine or an aliphatic amino acid
X3 is a negatively charged amino acid

5. Composition comprising
- a thermostable DNA Polymerase
15 - at least one sort of a divalent cation, preferably Mg2+
- deoxynucleotides
- a buffer
- a synthetic peptide according to claims 1-4

6. Composition according to claim 5, further comprising at least one nucleic acid
20 compound.

7. Kit comprising
- a peptide according to claims 1-4
- a thermostable DNA Polymerase

8. Method for amplification of a specific target nucleic acid comprising the steps of
25 - providing a sample suspected to contain said target nucleic acid
- adding a composition according to claims 5-6
- performing a nucleic acid amplification reaction

9. Method according to claim 8, characterized in that said nucleic acid amplification reaction is a Polymerase Chain Reaction which is monitored in real time

10. Method according to claims 8-9, characterized in that the amplification product generated by said amplification is subjected to a melting curve analysis.

5

AMENDED CLAIMS

received by the International Bureau on 5 June 2007 (05.06.2007)

1. Synthetic peptide having a length of not more than 30 amino acids comprising a divalent cation binding site selected from a group consisting of

   H-DIET-NH2
   H-DIETDIET-NH2
   H-DIETDIETDIET-NH2
   and H-FDGDFDGD-NH2

2. Composition comprising
   - a thermostable DNA Polymerase
   - at least one sort of a divalent cation, preferably Mg2+
   - deoxynucleotides
   - a buffer
   - a synthetic peptide having a length of not more than 30

3. Composition according to claim 2, further comprising at least one nucleic acid compound.

4. Kit comprising
   - a peptide according to amino acids comprising a divalent cation binding siH
   - a thermostable DNA Polymerase

5. Method for amplification of a specific target nucleic acid comprising the steps of
   - providing a sample suspected to contain said target nucleic acid
   - adding a composition according to claims 2-3
   - performing a nucleic acid amplification reaction

6. Method according to claim 5, characterized in that said nucleic acid amplification reaction is a Polymerase Chain Reaction which is monitored in real time

AMENDED SHEET (ARTICLE 19)

7. Method according to claims 5-6, characterized in that the amplification product generated by said amplification is subjected to a melting curve analysis.

Fig. 1

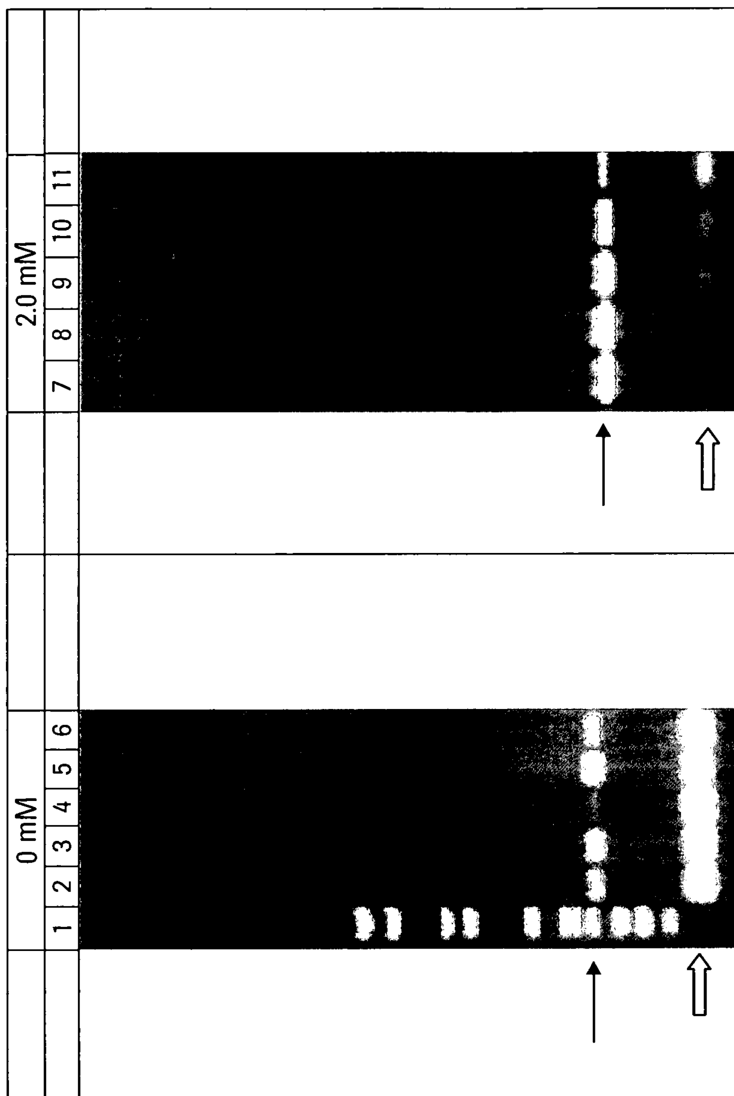


Fig. 2

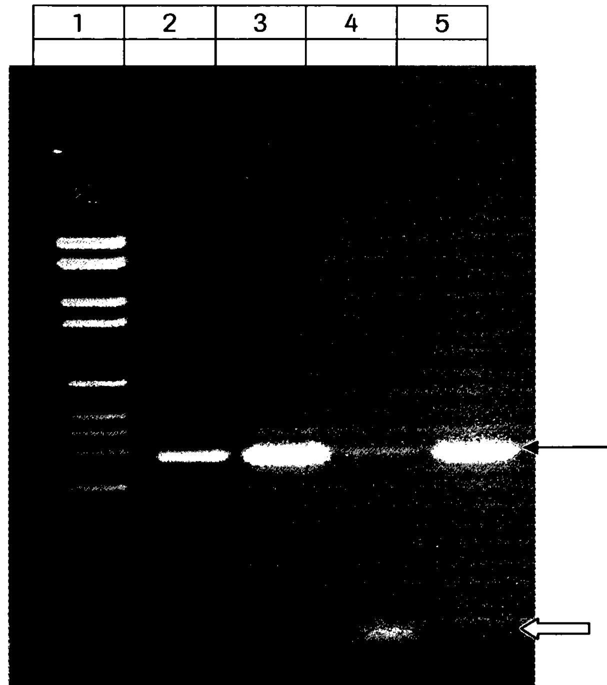


Fig. 3a

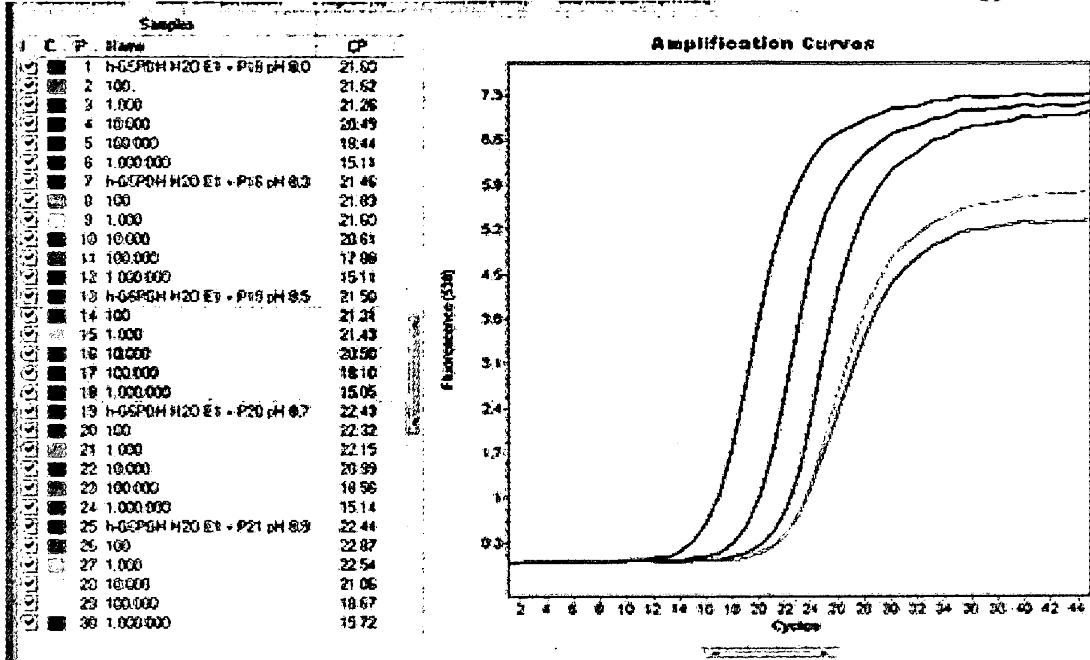


Fig. 3b

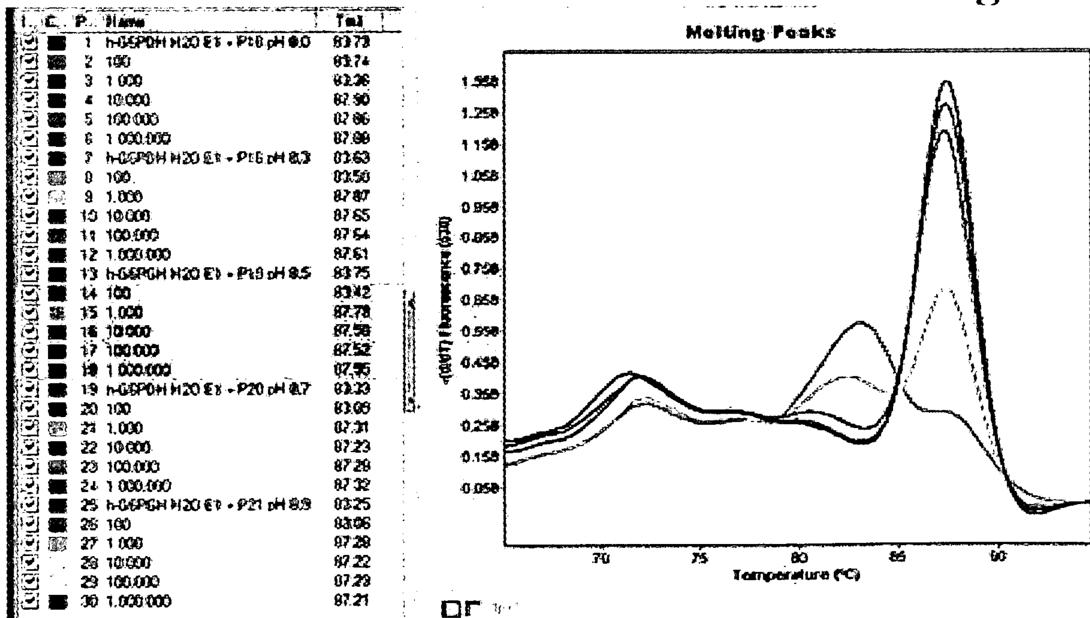


Fig. 3c

Samples				
I...	C...	P...	Name	CP
☑	■	1	h-G6PDH H2O E1 + P16 + DIET NH4	20.83
☑	■	2	100	21.09
☑	■	3	1.000	21.07
☑	■	4	10.000	20.55
☑	■	5	100.000	18.24
☑	■	6	1.000.000	15.05
☑	■	7	h-G6PDH H2O E1 + P16 + DIET TFA	26.49
☑	■	8	100	26.00
☑	□	9	1.000	24.81
☑	■	10	10.000	22.56
☑	■	11	100.000	18.98
☑	■	12	1.000.000	15.74
☑	■	13	h-G6PGH H2O E1 + P19 + DIET NH4	21.86
☑	■	14	100	22.14
☑	□	15	1.000	21.90
☑	■	16	10.000	21.23
☑	■	17	100.000	18.66
☑	■	18	1.000.000	15.60
☑	■	19	h-G6PDH H2O E1 + P19 + DIET TFA	27.66
☑	■	20	100	26.46
☑	■	21	1.000	25.51
☑	■	22	10.000	22.77
☑	■	23	100.000	19.52
☑	■	24	1.000.000	15.88

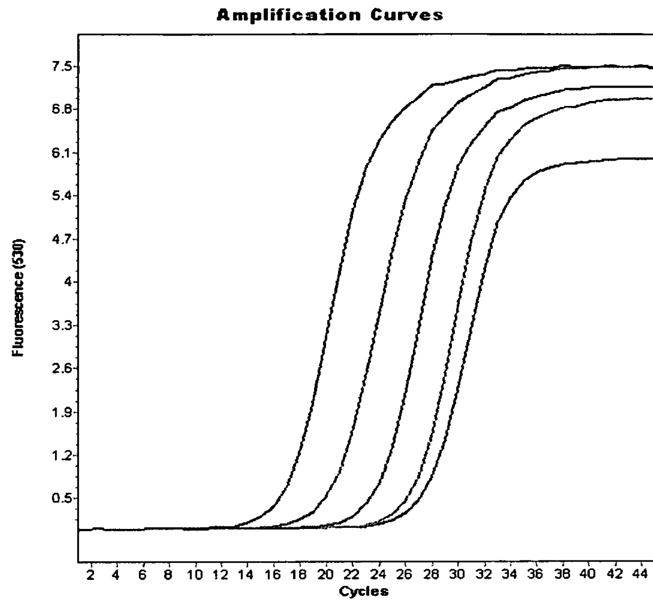
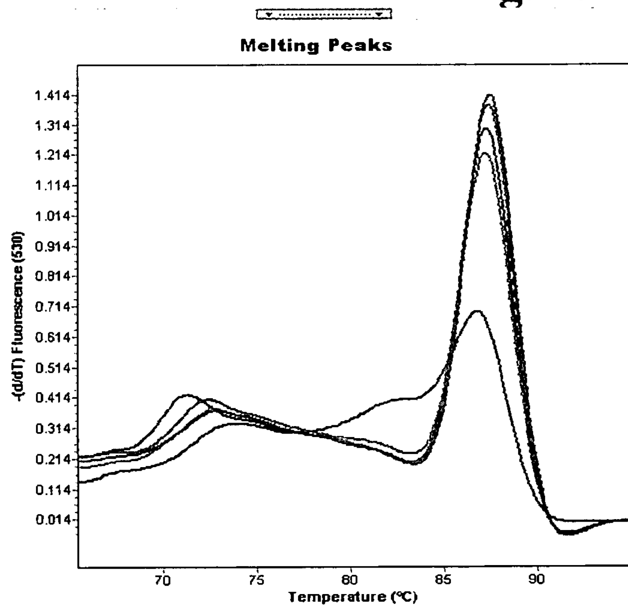


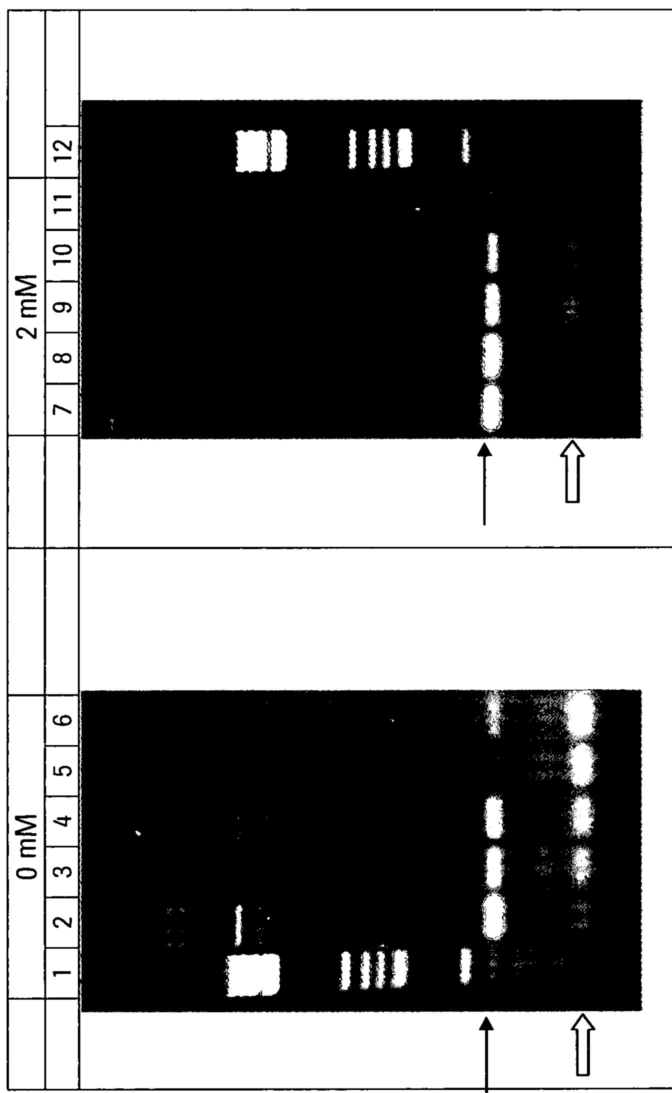
Fig. 3d

Samples				Melting
I...	Co...	Pos	Name	Tm1
☑	■	1	h-G6PDH H2O E1 + P16 + DIET NH4	83.86
☑	■	2	100	83.81
☑	■	3	1.000	83.29
☑	■	4	10.000	88.11
☑	■	5	100.000	88.03
☑	■	6	1.000.000	88.07
☑	■	7	h-G6PDH H2O E1 + P16 + DIET TFA	82.91
☑	■	8	100	87.71
☑	□	9	1.000	87.60
☑	■	10	10.000	87.57
☑	■	11	100.000	87.62
☑	■	12	1.000.000	87.61
☑	■	13	h-G6PGH H2O E1 + P19 + DIET NH4	83.63
☑	■	14	100	83.43
☑	□	15	1.000	83.19
☑	■	16	10.000	88.02
☑	■	17	100.000	87.86
☑	■	18	1.000.000	87.93
☑	■	19	h-G6PDH H2O E1 + P19 + DIET TFA	82.96
☑	■	20	100	87.25
☑	■	21	1.000	87.50
☑	■	22	10.000	87.52
☑	■	23	100.000	87.55
☑	■	24	1.000.000	87.61



4 / 5

Fig. 4



PCT/EP2007/001585

Diagnostics

European Patent Office

80298 München

Penzberg, June 26, 2007-HIL/SM
European Patent Application No. PCT/EP2007/001585
Applicant: Roche Diagnostics GmbH
Applicant's Ref.: 23634 WO-HIL

In response to the communication dated May 29, 2007:

On the basis of rule 91.1b PCT, applicant requests to correct figures 3a and b of the application referenced above. Figures 3a and b as filed on March 16, 2007, shall replace figures 3a and b as originally filed.

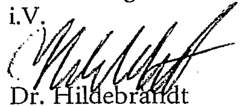
The new figures are different from the original figures only with respect to the fact that one column indicating either cp values or melting temperatures have been removed. This column, however, does not contain any relevant information with respect to the disclosure of the claimed invention.

If you have any questions on this matter, please do not hesitate to contact Dr. Martin Hildebrandt, Patent Department (TR-E), at phone +49 (8856) 60 3974, fax +49 (8856) 60 3451, or E-mail penzberg.patents@roche.com.

Very truly yours,

Roche Diagnostics GmbH

i.V. i.V.



Dr. Hildebrandt Dr. Misera

23634 WO 070626 to EPA RESPONSE (Amended Fig.).doc

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2111730

INTERNATIONAL SEARCH REPORT

International application No
PCT/EP2007/001585

A. CLASSIFICATION OF SUBJECT MATTER
INV. C12Q1/68 C07K5/113 C07K7/06 C07K7/08

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
C12Q

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

EPO-Internal, WPI Data, PAJ, BIOSIS, EMBASE

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	CIERNIEWSKI ET AL.: "Characterization of Cation-Binding Sequences in the Platelet Integrin GPIIb-IIIa (?IIb?3) by Terbium Luminescence" BIOCHEMISTRY, vol. 33, no. 40, 11 October 1994 (1994-10-11), pages 12238-12246, XP002383780 table 1 table 2 -----	1-4

-/--

[X] Further documents are listed in the continuation of Box C.

[X] See patent family annex.

* Special categories of cited documents :

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
4 May 2007	18/05/2007

Name and mailing address of the ISA/	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL – 2280 HV Rijswijk Tel. (+31-70) 340-2040, Tx. 31 651 epo nl, Fax: (+31-70) 340-3016	Santagati, Fabio

Form PCT/ISA/210 (second sheet) (April 2005)

C(Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	ZHAO ET AL.: "Gas-Phase Fragmentation of Anionic Complexes and Alkaline Earth Metal Ions: Structure-Specific Side-Chain Interactions" JOURNAL OF THE AMERICAN CHEMICAL SOCIETY, vol. 115, no. 7, 7 April 1993 (1993-04-07), pages 2854-2863, XP002383781 the whole document -----	1-4
X	XU C R ET AL: "STRUCTURAL AND ICAM-1-DOCKING PROPERTIES OF A CYCLIC PEPTIDE FROM THE I-DOMAIN OF LFA-1: AN INHIBITOR OF ICAM-1/LFA-1-MEDIATED T-CELL ADHESION" JOURNAL OF BIOMOLECULAR STRUCTURE & DYNAMICS, ADENINE PRESS, NEW YORK, NY, US, vol. 19, no. 5, April 2002 (2002-04), pages 789-799, XP009034557 ISSN: 0739-1102 the whole document -----	1-4
A	US 6 403 341 B1 (BARNES WAYNE M ET AL) 11 June 2002 (2002-06-11) cited in the application the whole document -----	5,7,8

INTERNATIONAL SEARCH REPORT

Information on patent family members

International application No
PCT/EP2007/001585

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
US 6403341 B1	11-06-2002	US 2003082567 A1	01-05-2003

Form PCT/ISA/210 (patent family annex) (April 2005)

(12) UK Patent Application (19) GB (11) 2 416 352 (13) A

(43) Date of A Publication 25.01.2006

(21) Application No: 0416293.9

(22) Date of Filing: 21.07.2004

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(51) INT CL:
C12N 9/00 (2006.01)

(52) UK CL (Edition X):
C3H HHX1 H525 H529

(56) Documents Cited:
US 5858650 A
Arch. Biochem. Biophys., Vol.304, 1993, Korge, P. et al., "The effect of changes in iron redox states...", pp.420-428
J. Mol. Cell Cardiol., Vol.26, 1994, Korge, P. et al., "Iron effects on myocardial enzymes...", pp.151-162

(58) Field of Search:
INT CL^7 **C12Q**
Other: **Online: EPODOC, WPI, PAJ, BIOSIS, MEDLINE**

(54) Abstract Title: **Hot start of enzymatic reaction**

(57) The present invention provides processes for controlling the start of an enzymatic reaction, which is catalysed by a metal ion dependent enzyme. The required metal ion is generated by a redox reaction initiated by heating a metal compound having a metal atom or metal ion with a redox agent. Also provided are kits for controlling the start of an enzymatic reaction. The processes and kits of the invention are useful for improving the specificity and performance of PCR.

GB 2 416 352 A

Original Printed on Recycled Paper

FIGURE 1

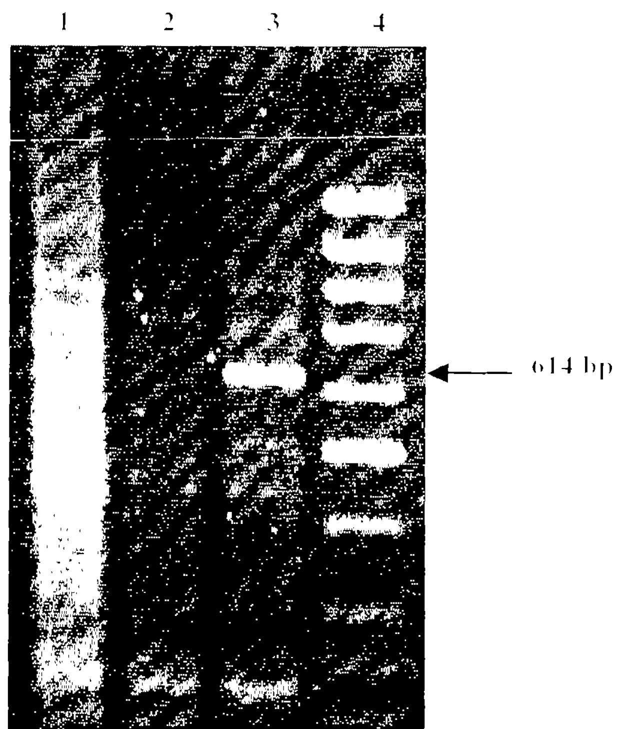


FIGURE 2

2416352

A METHOD FOR PERFORMING THE HOT START OF ENZYMATIC REACTIONS

FIELD OF THE INVENTION

5

The present invention provides processes and kits for controlling the start of an enzymatic reaction. A metal-ion dependent enzyme catalyses the enzymic reaction, with the required metal ion generated by a redox reaction. The 10 processes of the present invention are useful for improving the specificity and performance of PCR.

BACKGROUND

15 The present invention provides a method for performing an enzymatic reaction, which is catalyzed by a metal-ion dependent enzyme (*e.g.*, a restriction endonuclease, a DNA ligase, a reverse transcriptase or a DNA dependent DNA polymerase).

20

In biomolecular processes it is often important to control the activity of an enzyme. This is particularly the case with DNA polymerase enzymes used for the polymerase chain reaction (PCR). PCR reactions often involve the use of a divalent metal 25 ion-dependent heat-resistant DNA polymerase enzyme (such as *Taq* DNA polymerase) in a multi-cycle process employing several alternating heating and cooling steps to amplify DNA (U.S. Pat. Nos. 4,683,202 and 4,683,195). First, a reaction mixture is heated to a temperature sufficient to denature the double 30 stranded target DNA into its two single strands. The temperature of the reaction mixture is then decreased to allow specific oligonucleotide primers to anneal to their respective complementary single stranded target DNAs. Following the annealing step, the temperature is raised to the temperature 35 optimum of the DNA polymerase being used, which allows incorporation of complementary nucleotides at the 3' ends of

the annealed oligonucleotide primers thereby recreating double stranded target DNA. Using a heat-stable DNA polymerase, the cycle of denaturing, annealing and extension may be repeated as many times as necessary to generate a desired product, without the addition of polymerase after each heat denaturation. Twenty or thirty replication cycles can yield up to a million-fold amplification of the target DNA sequence ("Current Protocols in Molecular Biology," F.M. Ausubel et al. (Eds.), John Wiley and Sons, Inc., 1998).

Although PCR technology has had a profound impact on biomedical research and genetic identity analysis, amplification of non-target oligonucleotides and mispriming on non-target background DNA, RNA, and/or the primers themselves, still presents a significant problem. This is especially true in diagnostic applications where PCR is carried out in a milieu of complex genetic backgrounds where the target DNA may be proportionately present at a very low level (Chou et al., Nucleic Acid Res., 20:1717-1723 (1992).

A chief problem is that even though the optimal temperature for *Taq* DNA polymerase activity is typically in the range of 62° - 72°C, significant activity can also occur between 20° - 37°C (W.M. Barnes, et al, U.S. Pat. No. 6,403,341). As a result, during standard PCR preparation at ambient temperatures, primers may prime extensions at non-specific sequences because only a few base pairs at the 3'-end of a primer which are complementary to a DNA sequence can result in a stable priming complex. As a result, competitive or inhibitory products can be produced at the expense of the desired product. Thus, for example, structures consisting only of primers, sometimes called "primer dimers" can be formed by *Taq* DNA polymerase activity on primers inappropriately paired with each other.

The probability of undesirable primer-primer interactions also increases with the number of primer pairs in a reaction, particularly in the case of multiplex PCR. Mispriming of template DNA can also result in the production of inhibitory products or "wrong bands" of various lengths. During PCR cycling, non-specific amplification of undesired products can compete with amplification of the desired target DNA for necessary factors and extension constituents, such as dNTPs, which can lead to misinterpretation of the assay. Given the sensitivity of *Taq* DNA polymerase and its propensity to progressively amplify relatively large amounts of DNA from any primed event, it is imperative to control *Taq* DNA polymerase activity to prevent production of irrelevant, contaminating DNA amplification products, particularly when setting up PCR reactions.

Undesirable PCR side reactions typically occur during PCR preparation at ambient temperatures. One approach for minimizing these side reactions involves excluding at least one essential reagent (dNTPs, Mg^{2+}, DNA polymerase or primers) from the reaction until all the reaction components are brought up to a high (*e.g.,* DNA denaturation) temperature; the idea is to prevent binding of primers to one another or to undesired target sequences (Erlich, et al, Science 252, 1643-1651, 1991; D'Aquila, et al, Nucleic Acids Res. 19, 3749, 1991). This is an example of a "physical" PCR hot-start approach where an essential component is physically withheld until a desired reaction temperature is reached.

Other hot-start approaches have been described that physically segregate the reaction components from each other to guarantee that DNA polymerase activity is suppressed during the period preceding PCR initiation. In this way, a physical segregation of a hot start can be achieved by using a wax barrier, such as the method disclosed in U.S. Pat. Nos. 5,599,660 and

5,411,876. See also Hebert et al., Mol. Cell Probes, 7:249-252 (1993); Horton et al., Biotechniques, 16:42-43 (1994).

Other hot-start approaches have been described that employ the 5 "chemical/biochemical hot-start" methods that utilize modified DNA polymerases reversibly activatable upon heating (*e.g.*, AMPLITAQ GOLD™ DNA POLYMERASE, PE Applied Biosystems) or monoclonal, inactivating antibodies against *Taq* DNA polymerase that are bound to the polymerase at ambient temperatures 10 (Scalice et al., J. Immun. Methods, 172: 147-163, 1994; Sharkey et al., Bio/Technology, 12:506-509, 1994; Kellogg et al., Biotechniques, 16: 1134-1137, 1994).

The aforementioned different PCR hot-start approaches have 15 multiple shortcomings. Physical hot-start methods are plagued by contamination problems, plugging up of pipet tips with wax or grease and increased heating times. Chemical/biochemical hot-start methods can damage the template DNA and can require prohibitively excessive amounts of expensive anti-Amplitaq™ 20 antibodies.

Accordingly, there is a need in the art for new PCR hot-start methods minimizing or eliminating the many problems or shortcomings associated with the prior art procedures. More 25 generally, there is a need for new approaches for controlling metal-ion dependent enzymes where controlled activity is desired.

SUMMARY OF INVENTION

The present invention provides processes and reaction kits for 30 initiating an enzymatic reaction catalysed by a metal ion-dependent enzyme.

A process of the invention may comprise the steps of:

a) providing a reaction mixture comprising

i) a metal compound having a metal atom or metal ion in a first oxidation state;
ii) a redox agent; and
iii) a metal ion-dependent enzyme;

5 b) heating the mixture of step (a) to react the metal compound with the redox agent in a redox reaction, thereby converting the metal atom or metal ion to a second oxidation state;

wherein, the metal ion-dependent enzyme is activated by the
10 metal atom or metal ion in the second oxidation state.

In one embodiment of the present invention, the first oxidation state of the metal atom or metal ion in the metal compound may be an oxidized state. The second oxidation state
15 of the metal atom or metal ion may be a reduced state. The redox agent is a reducing agent.

In an alternative embodiment, the first oxidation state of the metal atom or metal ion in the metal compound may be a reduced
20 state. The second oxidation state of the metal atom or metal ion may be an oxidized state. The redox agent is an oxidizing agent.

The redox reaction that generates the metal atom or metal ion
25 in a second oxidation state can occur in a controlled manner, depending on physical conditions. These conditions include temperature and incubation time. Preferably the reaction mixture is heated to a temperature greater than 50°C. In effect, the redox reaction can provide a controlled generation
30 of an essential metal ion and as a result, controlled initiation of an enzymatic process catalysed by a metal ion-dependent enzyme.

The metal atom or metal ion in the second oxidation state may
35 include a monovalent, divalent or polyvalent metal ion from

one of cobalt, manganese, cadmium, copper, iron, molybdenum, nickel or chromium. Preferably the metal atom or metal ion in the second oxidation state is a divalent ion. More preferably the metal ion in the second oxidation state is Co^{2+}.

5

The reaction generating the metal ion in the second oxidation state can be a redox reaction, such as a reduction of cobalt (III) to cobalt (II), or a similar reaction such as the reduction of iron (III) to iron (II), chromium (VI) or
10 chromium (III) to chromium (II), manganese (VII) or manganese (IV) to manganese (II).

In an embodiment of the present invention, the metal ion dependent enzyme may be selected from: a polymerase, a ligase,
15 an endonuclease, a kinase, a protease or a combination thereof. Preferably the enzyme is a thermostable enzyme such as DNA ligase or DNA polymerase. Where the enzyme is DNA polymerase, the enzyme is preferably *Taq* polymerase or a variant thereof.

20

The enzymatic reaction according to the present invention may comprise a PCR process.

A further embodiment of the present invention relates to kits
25 for use in the processes described above. A kit according to the present invention may comprise a number of components required to generate the metal atom or metal ion in a second oxidation state necessary for activating the metal ion-dependent enzyme and initiating the enzymatic process of the
30 invention. The kits may be suitable for use in PCR reactions. The reaction components may be stored separately to avoid unwanted initiation of a redox reaction.

Other features, aspects and advantages of the invention will
35 be, or will become, apparent to one with skill in the art upon

examination of the following figures and detailed description. It is intended that all such additional systems, features, aspects and advantages included within this description, are within the scope of the invention, and are protected by the following claims.

BRIEF DESCRIPTION OF THE FIGURES

These and other features, aspects, and advantages of the present invention will become better understood with regard to the following description, claims and accompanying drawings where:

FIG. 1 depicts an electrophoretic analysis of the PCR products obtained in Example 2 using conventional PCR with ordinary PCR-buffer containing Mg^{2+} (lane 1) or Co^{2+} (lane 2), or using PCR with controlled generation of Co^{2+} (lane 3). Lane 4 - DNA marker.

FIG. 2 depicts an electrophoretic analysis of DNA fragments obtained following restriction endonuclease digestion of pBR322 using Taq I as described in Example 3. The enzymatic reaction was performed with (lane 2) and without (lane 1) heat initiation of Co^{2+} generation. Lane 3 - positive control of endonuclease digestion in presence of Co^{2+} (conventional endonuclease digestion).

DETAILED DESCRIPTION

In order to provide a clear and consistent understanding of the specification and claims, the following definitions are provided.

"Metal atom or metal ion" is used herein to designate a metal atom or metal ion, which as a result of a redox reaction, undergoes a change in its oxidation state, thereby generating a metal ion necessary for activating a metal ion-dependent

enzyme. The metal atom or metal ion may be selected from atoms and ions of cobalt, manganese, cadmium, copper, iron, molybdenum, nickel or chromium. The metal ion may comprise a monovalent, divalent or polyvalent metal ion.

5

"Thermostable", "thermally stable" and "heat-stable" are used interchangeably herein to describe enzymes, which can withstand temperatures up to at least 95°C for several minutes without becoming irreversibly denatured. Typically, such

10 enzymes have an optimum temperature above 45°C, preferably between 50° to 75°C.

"Hot start" refers to the method of initiating an enzymic reaction by heating components of the reaction. The reaction

15 components may be heated to a specific temperature or to a range of temperatures.

The term "redox" refers to reduction-oxidation, a term that is well known in the art, in which reduction is gain of electrons

20 and oxidation is loss of electrons.

A "metal compound" describes a metal atom or ion in combination with another element or compound, for example, in combination with chlorine or sulphate to give a metal chloride

25 or metal sulphate. Formation of the metal compound involves a chemical reaction. Also encompassed within this definition are metal complexes or coordination compounds in which other atoms or ligands are bound to a central metal ion. The ligands may be negatively charged or strongly polar groups.

30

A metal atom in a first oxidation state describes a metal atom in a compound, in which the atom has an overall charge of zero i.e. the number of electrons equals the number of protons. A metal atom in a second oxidation state describes a metal atom

35 which posses a different number of electrons to the number it

possessed in the first oxidation state i.e. the metal atom in a second oxidation state is a metal ion.

A metal ion in a first oxidation state describes a metal ion in a compound, in which the ion is in a reduced or oxidized state. A metal ion in a second oxidation state describes a metal ion which posses a different number of electrons to the number it possessed in the first oxidation state.

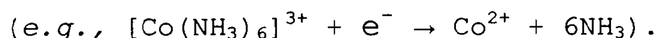
A redox reaction accounts for the transfer of electrons to or from the metal atom or metal ion in its first oxidation state to its second oxidation state. When the first oxidation state is a reduced state, the second oxidation state will be an oxidized state. When the first oxidation state is an oxidized state, the second oxidation state will be a reduced state.

The present invention provides processes for performing a metal ion-dependent enzymatic reaction in which required metal ions arise as a result of a non-enzymatic redox reaction. Generation of the metal ion by the redox reaction is determined by physical conditions of the reaction, such as temperature and incubation time. Thus, the redox reaction can provide a controlled generation of an essential metal ion. By controlling the generation of the metal ion, the present invention provides a means for controlling enzymatic processes, including, but not limited to, the start of an enzymatic process.

The redox reaction may provide a controlled generation of a metal ion, such as Co^{2+}. Preferably the redox reaction is the reduction of cobalt (III) to cobalt (II). For controlled generation of other metal ions, such as Fe^{2+}, Cr^{2+} or Mn^{2+}, similar reactions can be used (*e.g.*, reactions of reduction of iron (III) to iron (II), chromium (VI) or chromium (III) to chromium (II), manganese (VII) or manganese (IV) to manganese

(II), and others). As a reducing agent in these reactions ascorbic acid may be used, or potassium or sodium iodide, potassium or sodium thiosulfate or other reactants.

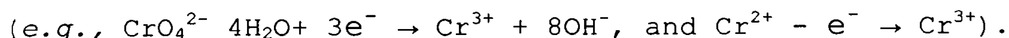
Preferred chemical reactions for generation of Co^{2+} as a metal ion for use with cobalt-dependent enzymes, include, but are not limited to reactions of reduction of cobalt (III) to cobalt (II)


(*e.g.*, $[Co(NH_3)_6]^{3+} + e^- \rightarrow Co^{2+} + 6NH_3$).

Preferred chemical reactions for generation of Mn^{2+} as a metal ion for use with manganese-dependent enzymes, include, but are not limited to reactions of reduction of manganese (VII) or manganese (IV) to manganese (II) (*e.g.*, $MnO_4^- + 4H_2O + 5e^- \rightarrow Mn^{2+} + 8OH^-$).

Preferred chemical reactions for generation of Cr^{2+} as a metal ion for use with chrome-dependent enzymes, include, but are not limited to reactions of reduction of chromium (VI) or chromium (III) to chromium (II) (*e.g.*, CrO_4^{2-} $4H_2O + 4e^- \rightarrow Cr^{2+} + 8OH^-$, or $Cr^{3+} + e^- \rightarrow Cr^{2+}$).

Preferred chemical reactions for generation of Cr^{3+} as a metal ion for use with chrome-dependent enzymes, include, but are not limited to reactions of reduction of chromium (VI) to chromium (III) and oxidation of chromium (II) to chromium (III)


(*e.g.*, CrO_4^{2-} $4H_2O + 3e^- \rightarrow Cr^{3+} + 8OH^-$, and $Cr^{2+} - e^- \rightarrow Cr^{3+}$).

Preferred chemical reactions for generation of Fe^{2+} as a metal ion for use with iron-dependent enzymes, include, but are not limited to reactions of reduction of iron (III) to iron (II) (*e.g.*, $Fe^{3+} + e^- \rightarrow Fe^{2+}$).

Preferred chemical reactions for generation of Fe^{3+} as a metal ion for use with iron-dependent enzymes, include, but are not limited to reactions of oxidation of iron (II) to iron (III) (*e.g.*, $Fe^{2+} - e^{-} \rightarrow Fe^{3+}$).

5

Preferred chemical reactions for generation of Cu^{2+} as a metal ion for use with copper-dependent enzymes, include, but are not limited to reactions of oxidation of copper (I) to copper (II) (*e.g.*, $Cu^{+} - e^{-} \rightarrow Cu^{2+}$)

10

Preferred chemical reactions for generation of Cu^{+} as a metal ion for use with copper-dependent enzymes, include, but are not limited to reactions of reduction of copper (II) to copper (I) (*e.g.*, $Cu^{2+} + e^{-} \rightarrow Cu^{+}$).

15

Preferred chemical reactions for generation of Ni^{2+} as a metal ion for use with nickel-dependent enzymes, include, but are not limited to reactions of reduction of nickel (III) to nickel (II) (*e.g.*, $Ni^{3+} + e^{-} \rightarrow Ni^{2+}$, or $Ni_2O_3 + 3H_2O + 2e^{-} \rightarrow$
20 $2Ni^{2+} + 6OH^{-}$).

Preferred metal compounds of cobalt (III) for use in redox reaction of Co^{2+} generation include, but are not limited to cobalt (III) complex compounds such as $[Co(NH_3)_6]Cl_3$,
25 $Na_3[Co(CN)_6]$ and others.

Preferred metal compounds of manganese (VII) and manganese (IV) for use in redox reaction of Mn^{2+} generation include, but are not limited to compounds such as $KMnO_4$, $NaMnO_4$, MnO_2,
30 $MnO(OH)_2$, and others.

Preferred metal compounds of chromium (VI) and chromium (III) for use in redox reaction of Cr^{2+} generation include, but are

not limited to compounds such as K_2CrO_4, $(NH_4)_2CrO_4$, $Cr_2(SO_4)_3$, $CrCl_3$, $Cr(OH)_3$, $Cr(NO_3)_3$ and others.

Preferred metal compounds of chromium (VI) and chromium (II) for use in redox reaction of Cr^{3+} generation include, but are not limited to compounds such as K_2CrO_4, $(NH_4)_2CrO_4$, $CrCl_2$, and others.

Preferred metal compounds of iron (III) for use in redox reaction of Fe^{2+} generation include, but are not limited to compounds such as $NH_4Fe(SO_4)_2$, $FeCl_3$, $Fe(NO_3)_3$, $Fe_2(SO_4)_3$and others.

Preferred metal compounds of iron (II) for use in redox reaction of Fe^{3+} generation include, but are not limited to compounds such as $(NH_4)_2Fe(SO_4)_2$, $FeCl_2$, $FeSO_4$ and others.

Preferred metal compounds of copper (I) for use in redox reaction of Cu^{2+} generation include, but are not limited to compounds such as CuCl, CuI, CuSCN and others.

Preferred metal compounds of copper (II) for use in redox reaction of Cu^{+} generation include, but are not limited to compounds such as $CuCl_2$, $CuBr_2$, $CuSO_4$ and others.

Preferred metal compounds of nickel (III) for use in redox reaction of Ni^{2+} generation include, but are not limited to compounds such as $CuCl_2$, $CuBr_2$, $CuSO_4$ and others.

The above mentioned redox reactions, which provide for generation of an essential metal-ion and, as a result, for the start of a metal-ion dependent enzymatic process, can be initiated by heating a reaction mixture to a temperature over 50°C. Thus, the metal-ion dependent enzymatic process can be started in a controlled manner after heating the reaction

mixture, thereby providing the hot-start of the enzymatic process.

The method of the invention may be applied to initiate or hot start metal-ion dependent enzymatic reactions which are catalyzed by DNA- and RNA-dependent DNA-polymerases, restriction endonucleases, DNA- and RNA-ligases, kinases, proteinases, and other metal-ion dependent enzymes. Particularly, the present invention can be used to initiate a PCR process.

The process of the present invention can increase the specificity of PCR reactions by preventing activation of a thermostable DNA polymerase (*e.g.* Taq DNA polymerase) at lower temperatures, while promoting temperature-dependent generation of divalent metal ions (*e.g.*, generation of Co^{2+} or Mn^{2+} at 60-98°C) and selection of specifically bound primers for DNA polymerase-catalyzed extension.

The PCR processes employ heat-stable DNA polymerase enzymes. These enzymes (*e.g.*, Taq, Tth or Pfu DNA polymerase) are divalent metal ion-dependent enzymes. These polymerases require the presence of Mg^{2+}, or Co^{2+}, or Mn^{2+} as a metal ion cofactor for activation. In order to perform a hot-start PCR by the method of the present invention, a reaction that generates Co^{2+} ions by reduction of cobalt (III) to cobalt (II) can be used.

Preferred reducing chemical agents for reduction of cobalt (III) to cobalt (II) in redox reaction of Co^{2+} generation include, but are not limited to ascorbic acid, salts of ascorbic acid, hydroiodic acid, salts of hydroiodic acid such as potassium, sodium or ammonium iodide, potassium thiosulphate and sodium thiosulphate.

In order to perform a hot-start PCR, the redox reaction between hexamminecobalt (III) chloride and ascorbic acid can be used. Under PCR conditions, this redox reaction generates Co^{2+} ions only at temperatures over 50°C. Thus, the enzymatic process (PCR) is initiated by the redox reaction only after heating the reaction mixture to a temperature above 50°C. As a result, the specificity of PCR is enhanced.

In a similar, the reduction-oxidation reaction between potassium permanganate ($KMnO_4$) and ascorbic acid ($C_6H_8O_6$) may be used, in order to perform PCR process. Under PCR conditions, this reduction-oxidation reaction generates Mn^{2+} ions.

Metal ion-dependent enzymes that may be controlled in accordance with the present invention include a variety of enzyme members or species defined by the several generic enzyme classes, including DNA polymerases, RNA polymerases, reverse transcriptases, DNA ligases, endonucleases, restriction endonucleases, kinases, and proteases. Metal-ion dependent enzymes may originate from a wide variety of animal, bacterial or viral sources, and may be synthesized from native genetic structures or from variants genetically modified by *e.g.*, mutagenesis or genetically modified to express fusion proteins, carrying multiple, distinct functional domains.

Additional examples of metal-ion dependent enzymes include DNA polymerases, such as Klenow fragment and DNA Pol I; reverse transcriptases (RT), such as AMV RT and MMLV RT; most restriction endonucleases; ribonucleases, such as RNase H; and topoisomerases, such as Topoisomerase I.

Many enzymes can alternatively use a few different metal ions. For example, RNA polymerases, such as RNA polymerase I or T7-, SP6-, and T4 RNA polymerases can use Mg^{2+} or Mn^{2+}. DNase I can

utilize a variety of different metal ions, including Mg^{2+}, Mn^{2+}, Ca^{2+}, Co^{2+} or Zn^{2+}.

Enzymes for use in the present invention may be preferably selected or engineered on the basis of retaining enzymatic stability under a range of reaction conditions required by generation of ionic enzymatic reactants, including high temperatures and/or various pH conditions (high/low, etc.). Particularly preferred enzymes include thermostable and/or pH resistant enzymes.

Thermostable enzymes may be isolated from thermophilic bacterial sources (*e.g.,* thermophilic genus *Thermus*) or they may be isolated and prepared by means of recombination. Representative species of the *Thermus* genus include *T. aquaticus, T. thermophilus, T. rubber, T. filiformis, T. brockianus and T. scotoductus.* The thermostable enzymes for use in the present invention may be derived from a broad range of enzyme types.

Examples of thermostable enzymes for use in the present invention, include, but are not limited to: thermostable DNA polymerases disclosed in *e.g.,* U.S. Pat. Nos. 4,889,818, 5,079,352, 5,192,674, 5,374,553, 5,413,926, 5,436, 149, 5,455,170, 5,545,552, 5,466,591, 5,500,363, 5,614,402, 5,616,494, 5,736,373, 5,744,312, 6,008,025, 6,027,918, 6,033,859, 6,130,045, 6,214,557; thermostable reverse transcriptases disclosed in *e.g.,* U.S. Pat. No. 5,998,195 and U.S. 2002/0090618; thermostable phosphatases disclosed in *e.g.,* U.S. Pat. Nos. 5,633,138, 5,665,551, 5,939,257; thermostable ligases disclosed in *e.g.,* U.S. Pat. Nos. 5,494,810, 5,506,137, 6,054,564 and 6,576,453; thermostable proteases disclosed in *e.g.,* U.S. Pat. Nos. 5,215,907, 5,346,820, 5,346,821, 5,643,777, 5,705,379, 6,143,517, 6,294,367, 6,358,726, 6,465,236; thermostable topoisomerases

disclosed in *e.g.,* U.S. Pat. Nos. 5,427,928 and 5,656,463; thermostable ribonucleases disclosed in *e.g.,* U.S. Pat. Nos. 5,459,055 and 5,500,370; thermostable beta-galactosidases disclosed in *e.g.,* U.S. Pat. Nos. 5,432,078 and 5,744,345;
5 thermostable restriction endonucleases, including *e.g.,* Acc III, Acs I/Apo I, Acy I, Bco I, BsaBI/BsiBI, BsaMI, BsaJI, BsaOI, BsaWI, BscBI, BscCI, BscFI, BseAI, BsiC1, BsiE1, BSi HKAJ, BsiLI, BsiMI, BsiQI, BsiWI, BsiXI, BsiZI, Bsi I, Bsm I, BsmAI, BsmBI, Bss, T11, Bsr1, BsrD1, Bsi711, BsiB1, BsiN1,
10 BsiU1, BsiY1, BsiZ1, Dsa 1, Mae 11, Mae 111, Mwo 1, Ssp B1, Taq I, Taq II, Taq52 I, Tfi I, Tru91, TspE1, TspRI, Tsp45 I, Tsp4C I, Tsp509 I, Tth111 II; Flap endonuclease disclosed in U.S. Pat. No. 6,251,649; and FLPe, a mutant, thermostable recombinase of Flp (Bucholz et al., Nature Biotechnology, Vol.
15 16, pp. 657-662, 1998).

Preferred metal ion-dependent enzymes include, but are not limited to thermally stable enzymes. Thermostable metal ion-dependent enzymes may include thermostable DNA polymerases,
20 RNA polymerases, reverse transcriptases, DNA ligases, endonucleases, restriction endonucleases, kinases, and proteases, including, but not limited to the aforementioned enzymes above. Thermally stable enzymes may be isolated from thermophilic bacterial sources or they may be isolated and
25 prepared by recombinant means.

Preferred DNA polymerases for use in PCR applications include thermally stable DNA polymerases and/or combinations thereof. Thermally stable DNA polymerases may include, but are not
30 limited to, *Thermus aquaticus* DNA polymerase and variations thereof such as N-terminal deletions of *Taq* polymerase, including the Stoffel fragment of DNA polymerase, Klentaq-235, and Klentaq-278; *Thermus thermophilus* DNA polymerase; *Bacillus caldotenax* DNA polymerase; *Thermus flavus* DNA polymerase;
35 *Bacillus stearothermophilus* DNA polymerase; and

archaebacterial DNA polymerases, such as *Thermococcus litoralis* DNA polymerase (also referred to as $Vent_R$®), Pfu, Pfx, Pwo, and $DeepVent_R$® or a mixture thereof. Other commercially available polymerases DNA polymerases include TaqLA or Expand High Fidelity^{Plus} Enzyme Blend (Roche); KlenTaqLA, KlenTaq1, TthLA (Perkin-Elmer), ExTaq® (Takara Shuzo); Elongase® (Life Technologies); Taquenase™ (Amersham), TthXL (Perkin Elmer); Advantage™ KlenTaq and Advantage™ Tth (Clontech); TaqPlus® and TaqExtender™ (Stratagene); or mixtures thereof.

In a further embodiment, the present invention includes methods for increasing the specificity of PCR. Preferably, the present invention provides processes and kits for performing a hot-start PCR. The processes and kits utilize the step of generating metal ions, to activate a DNA polymerase enzyme when the temperature of the reaction medium is raised to that enabling metal ion generation by the redox reaction. By performing a hot-start of PCR, the amplification specificity of the target DNA molecules is increased, with minimum or no formation of competitive or inhibitory products.

In a further embodiment, a kit is provided for use in a method of the present invention. Preferably the kit comprises a reaction buffer, a metal compound, an redox agent (e.g. a reducing agent) and a thermostable enzyme, whose activity is dependent on the metal ion in a second oxidation state. Where the thermostable enzyme is a DNA ligase, the kit may further comprise ATP and/or one or more synthetic oligonucleotides. Where the thermostable enzyme is a DNA polymerase, the kit may further comprise dNTPs and/or one or more synthetic oligonucleotides. Preferably the kit comprises a pair of synthetic oligonucleotides or more than one pair or synthetic oligonucleotides for use in a multiplexing PCR reaction. The reaction buffer may also comprise the metal compound.

To aid detection of a PCR product during each cycle of PCR, a technique known in the art as Real-Time PCR can be used. This relies on the detection and quantification of a signal from a fluorescent reporter, the level of which increases in direct proportion to the amount of PCR product being produced.

Therefore, the kit of the present invention may further comprise a fluorescent dye such as SYBR Green®, which binds double stranded DNA. However, since this reporter binds to any double stranded DNA in the reaction e.g. primer-dimers, an overestimation of the product amount may result. Alternatively, the kit may further comprise a reporter probe (e.g. TaqMan®) that contains a fluorescent dye and a quenching dye. These probes hybridize to an internal region of a PCR product and during PCR, when the polymerase enzyme replicates a template on which a reporter probe is bound, the 5' exonuclease activity of the polymerase cleaves the probe. This separates the fluorescent and quenching dyes resulting in a fluorescent signal. Molecular beacons, which also contain a fluorescent dye and a quenching dye, work on similar principle to TaqMan probes.

In order to prevent premature initiation of the process of the invention, the metal compound can be stored separately to the redox agent. Such storage may be by means of separate vials under conditions appropriate for the storage of reagents for use in PCR or a ligase chain reaction (LCR).

The present reaction composition can be applied to PCR processes as set forth in the Examples.

The principles, methodologies and examples described herein (and below) for controlling metal ion-dependent DNA polymerase

activity may be applied in an analogous fashion to control various types of metal ion-dependent enzymes described above.

The following examples illustrate aspects of the invention.

FIGURES

Figure 1

This figure depicts the electrophoretic analysis of the amplification products obtained when a 614-bp DNA fragment was amplified from 50 ng of *Gallus domesticus* genomic DNA for 30 cycles. PCR was performed in conventional conditions with ordinary PCR-buffer containing Mg^{2+} (lane 1) or Co^{2+} (lane 2). Lane 3 - PCR was performed using controlled generation of Co^{2+}. Lane 4 - DNA marker. Under these reaction conditions only the controlled generation of divalent ions provided a detectable amount of the desired product (lane 3). Compared to the conventional PCR procedures with Mg^{2+} (lane 1) and Co^{2+} (lane 2), fewer non-specific amplification products were obtained when using controlled generation of Co^{2+} (note the absence of non-specific amplification products in lane 3 compared to lane 1).

Figure 2

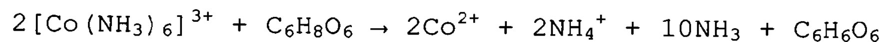
This figure is an electrophoretic analysis of DNA fragments obtained following restriction endonuclease digestion of pBR322 using Taq I as described in Example 3, indicating that controlled activation of restriction endonuclease activity can be achieved by controlled generation of divalent ions. The enzymatic reaction was performed with (lane 2) and without (lane 1) heat initiation of Co^{2+} generation (note the absence of digestion products in lane 1 compared to lane 2). Lane 3 - positive control of endonuclease digestion in presence of Co^{2+}.

EXAMPLES

Example 1

The Control of Co^{2+}-ions Chemical Generation by Changing Reaction Temperature.

Generation of Co^{2+} ions was performed by the reduction-oxidation reaction between hexamminecobalt (III) chloride and ascorbic acid. As a result of the reaction, cobalt (III) was reduced to cobalt (II), and Co^{2+}-ions were generated.



$$2[Co(NH_3)_6]^{3+} + C_6H_8O_6 \rightarrow 2Co^{2+} + 2NH_4^{+} + 10NH_3 + C_6H_6O_6$$

Generation of Co^{2+}-ions from $[Co(NH_3)_6]^{3+}$ ions is accompanied by the change of the solution color from yellow to pink. The change of color provides a possibility to monitor the reaction process and the Co^{2+} generation.

The reaction mixture contained: 10 mM hexamminecobalt (III) chloride ($[Co(NH_3)_6]Cl_3$); 20 mM ascorbic acid ($C_6H_8O_6$); 100 mM Tris-HCl, pH 9.0 at 25°C. Samples of the reaction mixture (500 µl) were incubated at 25°C, 40°C, 55°C, 70°C, and 85°C. The yellow color of the reaction mixture changed to pink color after the following incubations: 1.5 minutes at 85°C; 9 minutes at 70°C; and 80 minutes at 55°C. Incubations at 25°C and 40°C for 8 hours did not result in a change of color of the samples. Thus, the reaction of Co^{2+} generation can occur in a controlled manner by heating the reaction mixture.

Example 2

Increased Specificity of PCR Using Controlled Generation of Co^{2+} Compared to PCR Performed under Conventional Conditions (in presence of divalent ions)

A) Conventional PCR in presence of Mg^{2+}

A 614-bp DNA fragment was amplified from 50 ng of *Gallus domesticus* genomic DNA in 30 cycles: 95°C - 30 sec; 58°C - 30

sec; 72°C - 30 sec. The reaction mixture (50 µl) contained: 1.5 mM $MgCl_2$, 20 mM Tris-HCl (pH 9.0 at 25°C.), 50 mM NH_4Cl, 0.1% Triton X-100, 0.2 mM each dNTP, 25 pmol primer Pr1 (5'-attactcgagatcctggacaccagc), 25 pmol primer Pr2 (5'-attaggatcctgccctctcccca), and 5U Taq DNA polymerase.

B) Conventional PCR in presence of Co^{2+}

A 614 bp DNA fragment was amplified from 50 ng of *Gallus domesticus* genomic DNA in 30 cycles: 95°C - 30 sec; 58°C - 30 sec; 72°C - 30 sec. The reaction mixture (50 µl) contained: 1 mM $CoCl_2$, 20 mM Tris-HCl (pH 9.0 at 25°C.), 50 mM NH_4Cl, 0.1% Triton X-100, 0.2 mM each dNTP, 25 pmol primer Pr1 (5'-attactcgagatcctggacaccagc), 25 pmol primer Pr2 (5'-attaggatcctgccctctcccca), and 5U Taq DNA polymerase.

C) PCR using controlled generation of Co^{2+}

A 614 bp DNA fragment was amplified from 50 ng of *Gallus domesticus* genomic DNA in 30 cycles: 95°C - 30 sec; 58°C - 30 sec; 72°C - 30 sec. The reaction mixture (50 µl) contained: 1 mM hexamminecobalt (III) chloride ($[Co(NH_3)_6]Cl_3$), 2 mM ascorbic acid ($C_6H_8O_6$), 20 mM Tris-HCl (pH 9.0 at 25°C.), 50 mM NH_4Cl, 0.1% Triton X-100, 0.2 mM each dNTP, 25 pmol primer Pr1 (5'-attactcgagatcctggacaccagc), 25 pmol primer Pr2 (5'-attaggatcctgccctctcccca), and 5U Taq DNA polymerase.

Example 3

Control of Restriction Endonuclease Digestion

A) Controlling restriction endonuclease digestion by Co^{2+}generation

A 100 µl restriction enzyme digestion mixture (100 mM NaCl; 20 mM Tris-HCl (pH 8.5 at 25° C); 2 µg DNA pBR322; 5 U Taq I restriction endonuclease; 5 mM hexamminecobalt (III) chloride ($[Co(NH_3)_6]Cl_3$), 7 mM ascorbic acid ($C_6H_8O_6$)) was prepared. 50 µl samples were removed and placed into two reaction tubes. First tube was incubated at 47°C for 75 minutes. Second tube

was heated to 70°C for 10 minutes (for heat initiation of Co^{2+}generation), and then it was incubated at 47°C for 75 minutes.

B) Conventional restriction endonuclease digestion in
5 *presence of Co^{2+} (as a positive control of endonuclease digestion)*

A 100 µl restriction enzyme digestion mixture (100 mM NaCl; 20 mM Tris-HCl (pH 8.5 at 25° C); 2 µg DNA pBR322; 5 U Taq I restriction endonuclease; and 5 mM $CoCl_2$) was incubated at 47°C
10 for 75 minutes.

It is to be understood that the above-described methods are merely representative embodiments illustrating the principles of this invention and that other variations in the methods may
15 be devised by those skilled in the art without departing from the spirit and scope of this invention.

CLAIMS

1. A process for initiating an enzymatic reaction catalysed by a metal ion-dependent enzyme, comprising the steps of:

   a) providing a reaction mixture comprising:
      i) a metal compound having a metal atom or metal ion in a first oxidation state;
      ii) a redox agent; and
      iii) a metal ion-dependent enzyme;

   b) heating the mixture of step (a) to react the metal compound with the redox agent in a redox reaction, thereby converting said meal atom or metal ion to a second oxidation state;

wherein, the metal ion-dependent enzyme is activated by the metal atom or metal ion in the second oxidation state.

2. The process according to claim 1, where the metal compound comprises a metal atom or metal ion selected from atoms and ions of: manganese, cadmium, cobalt, copper, iron, molybdenum, nickel, and chromium.

3. The process according to claim 1 or claim 2, wherein the first oxidation state of the metal atom or metal ion is an oxidized state, the redox agent is a reducing agent and the second oxidation state is a reduced state.

4. The process according to claim 1 or claim 2, wherein the first oxidation state of the metal atom or metal ion is a reduced state, the redox agent is an oxidizing agent and the second oxidation state is an oxidized state.

5. The process according to any one of claims 1, 2 or 3, wherein the metal atom or metal ion in the second oxidation state is a divalent metal ion.

6. The process according to claim 5, wherein the divalent metal ion is Co^{2+}.

7. The process according to any one of claims 1 to 4, wherein the redox reaction is selected from:

a reduction of cobalt (III) to cobalt (II),
a reduction of manganese (VII) to manganese (II),
a reduction of manganese (IV) to manganese (II),
a reduction of manganese (III) to manganese (II),
a reduction of chrome (VI) to chrome (II),
a reduction of chrome (III) to chrome (II),
a reduction of iron (III) to iron (II),
a reduction of copper (II) to copper (I),
a reduction of nickel (III) to nickel (II),
a reduction of molybdenum (III) to molybdenum (II),
a reduction of molybdenum (VI) to molybdenum (II),
a reduction of molybdenum (VI) to molybdenum (III),
an oxidation of chromium (II) to chromium (III),
an oxidation of iron (II) to iron (III),
an oxidation of copper (I) to copper (II),
an oxidation of nickel (II) to nickel (III), and
an oxidation of cadmium (I) to cadmium (II).

8. The process according to any one of the preceding claims, wherein the redox agent is selected from: ascorbic acid, hydroiodic acid, potassium iodide, sodium iodide, ammonium iodide, potassium thiosulfate and sodium thiosulfate.

9. The process according to claim 6, wherein the redox reaction comprises a reaction between a compound of cobalt (III) and ascorbic acid.

10. The process according to claim 6, wherein the redox reaction comprises a reaction between a compound of cobalt (III) and hydroiodic acid.

11. The process according to claim 6, wherein the redox reaction comprises a reaction between hexamminecobalt (III) chloride and one of: ascorbic acid, sodium iodide, potassium iodide or ammonium iodide.

12. The process according to claim 6, wherein the redox reaction comprises a reaction between hexamminecobalt (III) chloride and ascorbic acid.

13. The process according to any one of the preceding claims, wherein in step (b), the reaction mixture is heated to a temperature greater than 50°C.

14. The process according to any one of the preceding claims, wherein the metal-ion dependent enzyme is: a polymerase, a ligase, an endonuclease, a kinase, a protease or a combination thereof.

15. The process according to claim 14, wherein the enzyme is a thermostable enzyme.

16. The process according to claim 15, wherein the enzyme is a thermostable DNA ligase.

17. The process according to claim 15, wherein the enzyme is a thermostable DNA polymerase.

18. The process according to claim 17, wherein the enzyme is *Taq* polymerase or a variant thereof.

19. The process according to any one of the preceding claims, wherein the enzymatic reaction is, or is part of, a PCR process.

20. A kit for use in the process of any one of claims 1 to 19, comprising a reaction buffer, a metal compound having a metal atom or ion in a first oxidation state, a redox agent and a thermostable enzyme.

21. The kit according to claim 20, wherein the first oxidation state of the metal ion is an oxidized state and the redox agent is a reducing agent.

22. The kit according to claim 20 or claim 21 further comprising ATP, and wherein the thermostable enzyme is a DNA ligase.

23. The kit according to claim 20 or claim 21 further comprising dNTPs, and wherein the thermostable enzyme is a DNA polymerase.

24. The kit according to claim 23, further comprising a fluorescent reporter suitable for use in Real-Time PCR.

25. The kit according to any one of claims 20 to 24 further comprising one or more synthetic oligonucleotides.

26. The kit according to any one of claims 20 to 25, wherein the redox agent and the metal compound are stored separately.

27. The kit according to any one of claims 20 to 25, wherein the redox agent and the thermostable enzyme are stored separately.

Application No:	GB0416293.9	**Examiner:**	Dr Jeremy Kaye
Claims searched:	1-27	**Date of search:**	27 September 2004

Patents Act 1977: Search Report under Section 17

Documents considered to be relevant:

Category	Relevant to claims	Identity of document and passage or figure of particular relevance
A	-	US 5858650 A (CELEBUSKI) see col.5, l.53 - col.11, l.17
A	-	Arch. Biochem. Biophys., Vol.304, 1993, Korge, P. et al., "The effect of changes in iron redox states...", pp.420-428
A	-	J. Mol. Cell Cardiol., Vol.26, 1994, Korge, P. et al., "Iron effects on myocardial enzymes...", pp.151-162

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Field of Search:

Search of GB, EP, WO & US patent documents classified in the following areas of the UKC[W] :

Worldwide search of patent documents classified in the following areas of the IPC[07]

C12Q

The following online and other databases have been used in the preparation of this search report

Online: EPODOC, WPI, PAJ, BIOSIS, MEDLINE

(12) INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(19) World Intellectual Property Organization
International Bureau

(43) International Publication Date
14 July 2011 (14.07.2011)

PCT

(10) International Publication Number
WO 2011/085160 A1

(51) International Patent Classification:
C12Q 1/68 (2006.01) *C12N 9/90* (2006.01)
C12N 9/12 (2006.01)

(21) International Application Number:
PCT/US2011/020459

(22) International Filing Date:
7 January 2011 (07.01.2011)

(25) Filing Language: English

(26) Publication Language: English

(30) Priority Data:
61/293,372 8 January 2010 (08.01.2010) US

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(81) Designated States *(unless otherwise indicated, for every kind of national protection available)*: AE, AG, AL, AM, AO, AT, AU, AZ, BA, BB, BG, BH, BR, BW, BY, BZ, CA, CH, CL, CN, CO, CR, CU, CZ, DE, DK, DM, DO, DZ, EC, EE, EG, ES, FI, GB, GD, GE, GH, GM, GT, HN, HR, HU, ID, IL, IN, IS, JP, KE, KG, KM, KN, KP, KR, KZ, LA, LC, LK, LR, LS, LT, LU, LY, MA, MD, ME, MG, MK, MN, MW, MX, MY, MZ, NA, NG, NI, NO, NZ, OM, PE, PG, PH, PL, PT, RO, RS, RU, SC, SD, SE, SG, SK, SL, SM, ST, SV, SY, TH, TJ, TM, TN, TR, TT, TZ, UA, UG, US, UZ, VC, VN, ZA, ZM, ZW.

(84) Designated States *(unless otherwise indicated, for every kind of regional protection available)*: ARIPO (BW, GH, GM, KE, LR, LS, MW, MZ, NA, SD, SL, SZ, TZ, UG, ZM, ZW), Eurasian (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European (AL, AT, BE, BG, CH, CY, CZ, DE, DK, EE, ES, FI, FR, GB, GR, HR, HU, IE, IS, IT, LT, LU, LV, MC, MK, MT, NL, NO, PL, PT, RO, RS, SE, SI, SK, SM, TR), OAPI (BF, BJ, CF, CG, CI, CM, GA, GN, GQ, GW, ML, MR, NE, SN, TD, TG).

Published:

— *with international search report (Art. 21(3))*

— *with sequence listing part of description (Rule 5.2(a))*

(54) **Title**: MATERIALS AND METHODS FOR ISOTHERMAL NUCLEIC ACID AMPLIFICATION

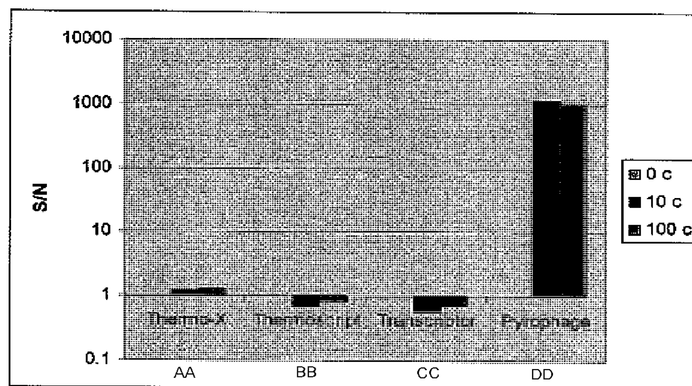

Fig. 3

(57) **Abstract**: A method for isothermal amplification of a target nucleic acid sequence is disclosed. The target nucleic acid is amplified by an enzyme with helicase activity and an enzyme with reverse transcriptase activity and DNA-dependant DNA polymerase activity. Also disclosed is a kit for isothermal amplification of a target nucleic acid sequence, including HPV nucleic acids. The kit comprises a first enzyme with helicase activity and a second enzyme having both reverse transcriptase activity and DNA-dependant DNA polymerase activity.

WO 2011/085160 A1

MATERIALS AND METHODS FOR ISOTHERMAL NUCLEIC ACID AMPLIFICATION

REFERENCE TO RELATED APPLICATIONS

[0001] This application claims priority to United States Provisional Patent Application Number 61/293,372, filed on January 8, 2010, which is incorporated herein by reference in its entirety.

BACKGROUND

[0002] Amplification of nucleic acids is widely used in research, forensics, medicine and agriculture. Polymerase chain reaction (PCR) is the most widely used method for *in vitro* DNA amplification. A PCR reaction typically utilizes two oligonucleotide primers that are hybridized to the 5' and 3' borders of the target sequence and a DNA-dependant DNA polymerase that extends the annealed primers by polymerizing deoxyribonucleotide-triphosphates (dNTPs) to generate double-stranded products. By raising and lowering the temperature of the reaction mixture (known as thermocycling), the two strands of the DNA product are separated and can serve as templates for the next round of annealing and extension, and the process is repeated.

[0003] In the past several years, other nucleic acid amplification methods have been developed that do not rely on thermocycling. These methods are broadly categorized as "isothermal target amplifications," owing to the fact that they do not rely on repeated cycles of temperature change to operate.

[0004] One such example is helicase-dependent amplification (HDA). *In vivo*, polymerases amplify nucleic acids with the aid of a variety of accessory proteins. One such class of accessory proteins is termed "helicases," which share the common characteristic of separating duplexed strands of nucleic acids into single strands, which are then accessible to polymerases for amplification. HDA mimics this general scheme *in vitro* by utilizing a helicase to generate single-stranded templates for primer hybridization and subsequent primer extension by a polymerase. By adding the helicase to the reaction mixture, repeated rounds of amplification can proceed without a need to repeatedly melt and re-anneal the primers to the templates. Accordingly, expensive thermocycling devices or tedious manual thermocycling can be avoided. In addition, HDA offers several advantages over other isothermal DNA amplification methods by having a simple reaction scheme and being a true isothermal reaction that can be performed at one temperature for the entire process.

[0005] One variation of PCR – termed reverse transcriptase PCR (RT-PCR) – is frequently used to measure gene expression, analyze RNA in samples, and synthesize modified complementary cDNA probes, among other uses. In the typical scheme, an enzyme having reverse transcriptase activity uses an RNA template to generate a complementary DNA strand (cDNA), which is then amplified via PCR. HDA may also be used to amplify the cDNA, in which case the process is termed reverse transcriptase HDA (RT-HDA). In either case, separate enzymes typically are used for each activity: a reverse transcriptase for generating a cDNA; a DNA-dependant DNA polymerase for amplifying the cDNA; and, in the case of HDA, a helicase for generating single stranded templates.

[0006] Unfortunately, reverse transcriptase enzymes can be highly error-prone, as they typically do not possess proof-reading abilities. Further, each enzyme added to the reaction mixture increases the potential necessity for different optimal temperatures, reaction conditions, reagents, etc. Thus, finding a set of enzymes and a set of conditions which produce high amounts of high fidelity DNA is often a difficult task. One way to simplify this task is to reduce the number of enzymes involved.

SUMMARY

[0007] Disclosed herein are materials and methods for performing an isothermal amplification of a target nucleic acid using an enzyme having both reverse transcriptase activity and DNA-dependant DNA polymerase activity.

[0008] One aspect is directed to a method for isothermal amplification of a target nucleic acid using a first enzyme having helicase activity and a second enzyme having both reverse transcriptase and DNA-dependant DNA polymerase activities.

[0009] Another aspect is directed to a method for isothermal amplification of a target RNA using a first enzyme having helicase activity and a second enzyme having both reverse transcriptase and DNA-dependant DNA polymerase activities.

[0010] Another aspect is directed to a method of isothermal amplification of a target RNA using a first enzyme having nick-inducing activity and a second enzyme having both reverse transcriptase and DNA-dependant DNA polymerase activities.

[0011] Another aspect is directed to a method of RT-HDA using a first enzyme having helicase activity and a second enzyme having both reverse transcriptase activity and DNA-dependant DNA polymerase activity.

[0012] Another aspect is directed to a method of identifying the presence of a human papilloma virus (HPV) in a sample comprising detecting a nucleic acid sequence of the HPV by using a first enzyme having a helicase activity and a second enzyme having both reverse transcriptase activity and DNA-dependant DNA polymerase activity.

[0013] Yet another aspect is directed a kit for isothermal amplification of a target nucleic acid comprising an first enzyme having a helicase activity and a second enzyme having both reverse transcriptase activity and DNA-dependant DNA polymerase activity.

[0014] In another aspect, a kit for isothermal amplification of a target RNA is provided comprising an enzyme having helicase activity and an enzyme having both reverse transcriptase activity and DNA-dependant DNA polymerase activity and does not comprise any other enzymes having DNA-dependant DNA polymerase activity.

[0015] Another aspect is directed to a kit for RT-HDA in which an enzyme having reverse transcriptase activity or an enzyme having polymerase activity, or both, are replaced by an enzyme having reverse transcriptase activity and DNA-dependant DNA polymerase activity.

[0016] Another aspect is directed to a kit for determining the presence and/or abundance of at least one human papilloma virus (HPV) in a sample comprising a first enzyme having helicase activity and a second enzyme having both reverse transcriptase activity and DNA-dependant DNA polymerase activity.

BRIEF DESCRIPTION OF THE FIGURES

[0017] FIG. 1 shows that PYROPHAGE 3173 is as effective as other reverse transcriptases in one-step, RT-HDA in the presence of Bst-polymerase. Amplification was performed in 25 μl for 75 minutes, utilizing Bst polymerase (2U) and uvrD helicase (1U). CtRNA was used as a target. The assay signal (Luminex MFI) for Thermoscript, Thermo-X, Transcriptor and PYROPHAGE is given for 10 and 100 RNA copies.

[0018] FIG. 2 shows that PYROPHAGE 3173 enzyme could perform as both a reverse transcriptase and a DNA-dependant DNA polymerase in a one step isothermal RT-HDA. Amplification was performed in 25 μL for 75 minute. The reaction mixture contained PYROPHAGE 3173 (2.5 U) either with Bst (bars labeled Bst +) or without the addition of Bst polymerase (bars labeled Bst-). Ct RNA (25 or 100 copies) was used as a target. Detection was performed on a Luminex and the results are presented as Signal/Noise.

[0019] FIG. 3 shows RT-HDA reactions using THERMO-X, THERMOSCRIPT, TRANSCRIPTOR, and PYROPHAGE 3173, in the absence of Bst-polymerase. Reaction conditions are the same as described in Figure 2.

[0020] FIG. 4 shows that PYROPHAGE 3173 enzyme could amplify DNA in the absence of Bst. Amplification was performed in 50 μL for 90 minute. Reactions contained either PYROPHAGE 3173 (5U) or Bst-polymerase (20U). HPV16 DNA was used as a target for standard tHDA assay with alkaline denaturation. Detection was performed on a Luminex and the results are presented as a Signal/Noise.

[0021] FIG. 5 shows the signal over noise (S/N) for RT-HDA reactions in which two HPV 16 RNA targets are amplified. Each bar represents S/N (y-axis) for each of the two targets after amplification with various primer concentrations. Primer concentrations and the amplified targets are indicated on the x-axis.

DETAILED DESCRIPTION

[0022] In one aspect, amplification of a target nucleic acid is accomplished by an enzyme having both reverse transcriptase activity and DNA-dependant DNA polymerase activity. This enzyme, with dual activities, is used as a substitute for, or in addition to, using a DNA-dependant DNA polymerase and/or a reverse transcriptase.

[0023] As used herein, "nucleic acid" refers to double stranded (ds) or single stranded (ss) DNA, RNA molecules or DNA-RNA hybrids. Double stranded nucleic acid molecules may be nicked or intact. The double stranded or single stranded nucleic acid molecules may be linear or circular. The duplexes may be blunt ended or have single stranded tails. The single stranded molecules may have secondary structure in the form of hairpins or loops and stems. The nucleic acid may be isolated from a variety of sources including the environment, food, agriculture, fermentations, biological fluids such as blood, milk, cerebrospinal fluid, sputum, saliva, stool, lung aspirates, swabs of mucosal tissues or tissue samples or cells. Nucleic acid samples may be obtained from cells, bacteria or viruses and may include any of: chromosomal DNA, extra chromosomal DNA including plasmid DNA, recombinant DNA, DNA fragments, messenger RNA, transfer RNA, ribosomal RNA, double stranded RNA or other RNAs that occur in cells, bacteria or viruses. The nucleic acid may be isolated, cloned or synthesized *in vitro* by means of chemical synthesis. Any of the above described nucleic acids may be subject to modification where individual nucleotides within the nucleic acid are chemically altered (for example, by

methylation). Modifications may arise naturally or by *in vitro* synthesis. The term "duplex" refers to a nucleic acid molecule that is double stranded in whole or part.

[0024] As used herein, the term “target nucleic acid” refers to any nucleic acid sequence that is intended to be amplified. The size of the target nucleic acid to be amplified may be, for example, in the range of about 50 bp to about 100 kb including a range of above 100 to 5000 bp. The target nucleic acid may be contained within a longer double stranded or single stranded nucleic acid. Alternatively, the target nucleic acid may be an entire double stranded or single stranded nucleic acid.

[0025] In one embodiment, the enzyme having both reverse transcriptase and DNA-dependant DNA polymerase activities is PYROPHAGE 3173. PYROPHAGE 3173 is described in U.S. Patent Application No. 12/089,221, published as U.S. Patent Application Publication No. 2008/0268498, the contents of which are incorporated in their entirety. PYROPHAGE 3173, is available from LUCIGEN Corporation, and is a thermostable bacteriophage enzyme that has an inherent 3’→5’ exonuclease (proofreading) activity, which results in high fidelity amplification. Because of this activity, it may be preferable to use phosphorothioate primers and minimal exposure of the target nucleic acid template and the primers prior to amplification. Alternatively, a mutant version may be used, in which the 3’→5’ exonuclease activity has been inactivated (PYROPHAGE 3173 Exo$^-$ mutant). PYROPHAGE 3173 also has strand-displacing activity that allows for DNA synthesis through double-stranded DNA. It also initiates efficiently at nicks and therefore DNA synthesis can be initiated either with primers or at a nick introduced by site-specific nicking enzymes. PYROPHAGE 3173 also has reverse transcription activity and thus can perform single-tube, single enzyme reverse transcription PCR on RNA templates. Because of this dual activity PYROPHAGE 3173 may be used in RT-HDA as a substitute for reverse transcriptase and DNA-dependant DNA polymerase. In addition, the higher thermostability of PYROPHAGE 3173 enzyme may allow higher RNA amplification rate.

[0026] In one embodiment, the target nucleic acid is amplified using an isothermal amplification. “Isothermal amplification” refers to amplification which occurs at a single temperature. This does not include the single brief time period (less than 15 minutes) at the initiation of amplification, which may be conducted at the same temperature as the amplification procedure or at a higher temperature.

[0027] In one embodiment, the isothermal amplification method is RT-HDA. Traditionally, three enzymes are used in RT-HDA: a reverse transcriptase, a helicase, and a DNA-dependant DNA polymerase. Reverse transcriptase (also known as RNA-dependent DNA polymerase), is an enzyme having a DNA polymerase activity that transcribes single stranded RNA (ssRNA) into a complementary single stranded DNA (cDNA) by polymerizing deoxyribonucleotide triphosphates (dNTPs). The same pyrophage enzyme may also polymerize the "second strand" of the cDNA making ds-DNA. This negates the use of two enzymes (reverse transcriptase and DNA-dependant DNA polymerase) in the traditional process of ds-DNA synthesis from ss-RNA. The helicase has an enzymatic activity that unwinds the ds-DNA for iterations (amplification) of primer-dependant DNA polymerization of top and bottom strands of ds-DNA. The DNA-dependant DNA polymerase then transcribes the cDNA into a complementary single stranded DNA by polymerizing dNTPs. This process repeats itself so that exponential amplification can be achieved at a single temperature without necessitating thermocycling. In one embodiment, RT-HDA is performed using a single enzyme to provide both the reverse transcriptase and DNA-dependant DNA polymerase activity.

[0028] As used herein, "HDA" refers to Helicase Dependent Amplification which is an *in vitro* method for amplifying nucleic acids by using a helicase preparation for unwinding a double stranded nucleic acid to generate templates for amplification.

[0029] As used herein, "Helicase" or "an enzyme with, or having, helicase activity" refers to any enzyme capable of unwinding a double stranded nucleic acid. For example, helicases are enzymes that are found in all organisms and in all processes that involve nucleic acid such as replication, recombination, repair, transcription, translation and RNA splicing. Any helicase that translocates along DNA or RNA in a 5'→3' direction or in the opposite 3'→5' direction may be used. This includes helicases obtained from prokaryotes, viruses, archaea, and eukaryotes or recombinant forms of naturally occurring enzymes as well as analogues or derivatives having the specified activity. Examples of naturally occurring DNA helicases include *E. coli* helicase I, II, III, & IV, Rep, DnaB, PriA, PcrA, T4 Gp41 helicase, T4 Dda helicase, T7 Gp4 helicases, SV40 Large T antigen, yeast RAD. Additional helicases that may be useful include RecQ helicase, thermostable UvrD helicases from *T. tengcongensis* and *T. thermophilus*, thermostable DnaB helicase from *T. aquaticus*, and MCM helicase from archaeal and eukaryotic organisms.

[0030] In another embodiment, the helicase is a thermostable helicase. Denaturation of nucleic acid duplexes can be accelerated by using a thermostable helicase preparation under incubation conditions that include higher temperature for example in a range of 45°C to 75°C. Performing HDA at high temperature using a thermostable helicase preparation and a thermostable polymerase may increase the specificity of primer binding, which can improve the specificity of amplification.

[0031] In a further embodiment, a plurality of different helicase enzymes is used in the amplification reaction. The use of a plurality of helicases may enhance the yield and length of target amplification in HDA under certain conditions where different helicases coordinate various functions to increase the efficiency of the unwinding of duplex nucleic acids. For example, a helicase that has low processivity but is able to melt blunt-ended DNA may be combined with a second helicase that has great processivity but recognizes single-stranded tails at the border of duplex region for the initiation of unwinding. In this example, the first helicase initially separates the blunt ends of a long nucleic acid duplex generating 5' and 3' single-stranded tails and then dissociates from that substrate due to its limited processivity. This partially unwound substrate is subsequently recognized by the second helicase that then continues the unwinding process with superior processivity. In this way, a long target in a nucleic acid duplex may be unwound by the use of a helicase preparation containing a plurality of helicases and subsequently amplified in a HDA reaction.

[0032] In a further embodiment, an accessory protein is included with the reaction mixture. "Accessory protein" refers to any protein capable of stimulating helicase activity. For example, *E. coli* MutL protein is an accessory protein for enhancing UvrD helicase activity. Accessory proteins are useful with selected helicases. However, unwinding of nucleic acids may be achieved by helicases in the absence of accessory proteins.

[0033] In another embodiment, at least one single-strand binding proteins (SSB) is included with the reaction mixture. Mesophilic helicases show improved activity in the presence of SSBs. In these circumstances, the choice of SSB is generally not limited to a specific protein. Examples of single strand binding proteins are T4 gene 32 protein, E. coli SSB, T7 gp2.5 SSB, phage phi29 SSB and truncated forms of these proteins. Thus, in certain embodiments, one or more SSBs may be added to an amplification reaction.

[0034] In yet another embodiment, at least one cofactor is provided. "Cofactors" refer to small-molecule agents that are required for the helicase unwinding activity. Helicase cofactors include nucleoside triphosphate (NTP) and deoxynucleoside triphosphate (dNTP) and magnesium (or other divalent cations). For example, ATP (adenosine triphosphate) may be used as a cofactor for UvrD helicase at a concentration in the range of 0.1 to 100 mM and preferably in the range of 1 to 10 mM (for example 3 mM). Similarly, dTTP (deoxythymidine triphosphate) may be used as a cofactor for T7 Gp4B helicase in the range of 1 to 10 mM (for example 3 mM).

[0035] In a further embodiment, the DNA-dependant DNA polymerase transcribes the cDNAs in a sequence-dependent amplification. "Sequence-dependent synthesis" or "sequence-dependent amplification" refers to amplification of a target sequence relative to non-target sequences present in a sample with the use of target-specific primers. As used herein, "target-specific primer" refers to a single stranded nucleic acid capable of binding to a pre-determined single stranded region on a target nucleic acid to facilitate polymerase dependent replication of the target nucleic acid to be selectively amplified.

[0036] In one embodiment, a pair of target-specific primers, one hybridizing to the 5'-flank of the target sequence and the other hybridizing to the 3'-flank of the target, are used to achieve exponential amplification of a target sequence.

[0037] In another embodiment, multiple pairs of target-specific primers can be utilized in a single reaction for amplifying multiple targets simultaneously using different detection tags in a multiplex reaction. Multiplexing is commonly used in single nucleotide polymorphism (SNP) analysis and in detecting pathogens.

[0038] Generally, suitable target-specific primer pairs are short synthetic oligonucleotides, for example having a length of 10 or more nucleotides and less than 50 nucleotides. Target-specific, oligonucleotide primer design involves various parameters such as string-based alignment scores, melting temperature, primer length and GC content. When designing a target-specific primer, one of the important factors is to choose a sequence within the target fragment that is specific to the nucleic acid molecule to be amplified. Another important factor is to calculate the melting temperature of a target-specific primer for the reaction. The melting temperature of a target-specific primer is determined by the length and GC content of that oligonucleotide. Preferably the melting temperature of a primer is about 10 to 30°C higher than the temperature at which primer hybridization and target amplification will take place.

[0039] "Primer hybridization" refers to binding of an oligonucleotide primer to a region of the single-stranded nucleic acid template under the conditions in which the primer binds only specifically to its complementary sequence on one of the template strands, not other regions in the template. The specificity of hybridization may be influenced by the length of the oligonucleotide primer, the temperature in which the hybridization reaction is performed, the ionic strength, and the pH of the reaction mixture.

[0040] Each target-specific primer hybridizes to each end of the target nucleic acid and may be extended in a 3'→5' direction by a polymerase using the target nucleotide sequence as a template. To achieve specific amplification, a homologous or perfect match target-specific primer is preferred. However, target-specific primers may include sequences at the 5' end which are non-complementary to the target nucleotide sequence(s). Alternatively, target-specific primers may contain nucleotides or sequences throughout that are not exactly complementary to the target nucleic acid.

[0041] The target-specific primers may include any of the deoxyribonucleotide bases A, T, G or C and/or one or more ribonucleotide bases, A, C, U, G and/or one or more modified nucleotide (deoxyribonucleotide or ribonucleotide) wherein the modification does not prevent hybridization of the primer to the nucleic acid or elongation of the target-specific primer or denaturation of double stranded molecules. Target-specific primers may be modified with chemical groups such as phosphorothioates or methylphosphonates or with non nucleotide linkers to enhance their performance or to facilitate the characterization of amplification products.

[0042] In general, the temperature of denaturation suitable for permitting specificity of target-specific primer-template recognition and subsequent annealing may occur over a range of temperatures, for example 20°C to 75°C. A preferred denaturation temperature may be selected according to which helicase is selected for the melting process. Tests to determine optimum temperatures for amplification of a nucleic acid in the presence of a selected helicase can be determined by routine experimentation by varying the temperature of the reaction mixture and comparing amplification products using gel electrophoresis.

[0043] The target-specific primers may be subject to modification, such as fluorescent or chemiluminescent-labeling, and biotinylation (for example, fluorescent tags such as amine reactive fluorescein ester of carboxyfluorescein). Other labeling methods include radioactive

isotopes, chromophores and ligands such as biotin or haptens, which while not directly detectable can be readily detected by reaction with labeled forms of their specific binding partners e.g. avidin and antibodies respectively. Such modifications can be used to detect the amplified products.

[0044] "Melting", "unwinding", or "denaturing" refer to separating all or part of two complementary strands of a nucleic acid duplex.

[0045] In a further embodiment, the DNA-dependant DNA polymerase transcribes the cDNA in a sequence-independent amplification. As used herein, "sequence-independent amplification" refers to any amplification performed by a DNA-dependant DNA polymerase that does not amplify a specific sequence. By way of example and not limitation, random primer mixtures or nick-inducing agents may be used to initiate sequence-independent amplification.

[0046] As used herein, "random primer mixture" refers to mixtures of short randomly generated oligonucleotide sequences.

[0047] As used herein, "nick-initiated polymerase activity" refers to polymerase activity in the absence of exogenous primers, which is initiated by single-strand breaks in the template. Synthesis initiates at the single-strand break in the DNA, rather than at the terminus of an exogenous synthetic primer. With nick-initiated synthesis, removal of primers is unnecessary, reducing cost, handling time and potential for loss or degradation of the product. In addition, nick-initiated synthesis reduces false amplification signals caused by self-extension of primers. The nicks may be introduced at defined locations, by using enzymes that nick at a recognition sequence, or may be introduced randomly in a target polynucleotide. As used herein, "nick-inducing agent" refers to any enzymatic or chemical reagent or physical treatment that introduces breaks in the phosphodiester bond between two adjacent nucleotides in one strand of a double-stranded nucleic acid. Examples of nick-inducing enzymes include Bpu10 I, BstNB I, Alw I, BbvC I, BbvC I, Bsm I, BsrD, and *E. coli* endonuclease I. In one embodiment, at least one nick-inducing enzyme is included as a replacement for a helicase in a reaction mixture. In another embodiment, at least one nick-inducing enzyme is added to a reaction mixture in addition to at least one helicase.

[0048] Other amplification reaction components may, in appropriate circumstances, include buffers, biomolecules, salts, urea, dimethyl-sulfoxide (DMSO), polyethylene glycol (PEG), magnesium, topoisomerases, accessory proteins, denaturating agents, cofactors, or

mixtures thereof. When primer-initiated amplification is desired, primers are added to the amplification reaction components.

[0049] Deoxyribonucleotide triphosphates dNTPs (i.e., dATP, dGTP, dCTP and dTTP), are added, which are used to build the new strand of DNA. ATP or TTP are added as an energy source. ATP or TTP is a commonly preferred energy source for highly processive helicases. On average one ATP molecule is consumed by a DNA helicases to unwind 1 to 4 base pairs. To amplify a longer target, more ATP may be consumed as compared to a shorter target. In these circumstances, it may be desirable to include a pyruvate kinase-based ATP regenerating system for use with the helicase. Thus, in certain embodiments, ATP or TTP or a combination or a pyruvate kinase-based ATP regenerating system may be added to the amplification reaction components.

[0050] Topoisomerases can be used in long HDA reactions to increase the ability of HDA to amplify long target amplicons. When a very long linear DNA duplex is separated by a helicase, the swivel (relaxing) function of a topoisomerase removes the twist and prevents over-winding. For example, *E. coli* topoisomerase I can be used to relax negatively supercoiled DNA by introducing a nick into one DNA strand. DNA gyrase (topoisomerase II) introduces a transient double-stranded break into DNA allowing DNA strands to pass through one another. Thus, in certain embodiments, a topoisomerase or a gyrase, or both may be added to the amplification reaction.

[0051] In a further embodiment, an amplified nucleic acid product may be detected by various methods including ethidium-bromide staining and detecting the amplified sequence by means of a label, such as, but not limited to: a radiolabel, a fluorescent-label, and an enzyme. For example HDA amplified products can be detected in real-time using fluorescent-labeled LUX Primers (Invitrogen Corporation, Carlsbad, Calif.), which are oligonucleotides designed with a fluorophore close to the 3' end in a hairpin structure. This configuration intrinsically renders fluorescence quenching capability without separate quenching moiety. When the primer becomes incorporated into double-stranded amplification product, the fluorophore is dequenched, resulting in a significant increase in fluorescent signal.

[0052] The present disclosure also encompasses a kit comprising an enzyme with helicase activity and an enzyme with both reverse transcriptase activity and DNA-dependant DNA polymerase activity. The kit may further comprise amplification reaction components

selected from, but not limited to, one or more of dNTPs, ATP, TTP, primers, magnesium, topoisomerases, SSB proteins, accessory proteins, denaturating agents, polyethylene glycol, cofactors, or mixtures thereof.

[0053] A further embodiment relates to a mixture comprising a nucleic acid that is a target for isothermal amplification. The nucleic acid target may be ssDNA, dsDNA, ssRNA, dsRNA, RNA-DNA hybrid, or a mixture of any of the above. The mixture comprising the target nucleic acid also comprises at least one enzyme with helicase activity and at least one enzyme with both reverse transcriptase activity and DNA-dependant DNA polymerase activity. The mixture comprising the target nucleic acid and the enzymes can also comprise one or more of the amplification reaction components previously described.

[0054] Another aspect is an amplified nucleic acid obtained by the amplification methods described. The amplified nucleic acid may be DNA or RNA.

[0055] In another aspect, a kit is provided for detecting an HPV RNA using an isothermal reverse transcriptase/amplification reaction, wherein the kit comprises at least one enzyme having both reverse transcriptase and DNA-dependant DNA polymerase activity. In one embodiment, the kit further comprises at least one enzyme having an activity selected from the group consisting of helicase activity and nick-inducing activity. In another embodiment, the kit further comprises at least one enzyme having a helicase activity and at least one enzyme having a nick-inducing activity. The kit may further comprise other reagents necessary for conducting the desired amplification, including but not limited to: buffers; biomolecules; salts; urea; dimethyl-sulfoxide (DMSO); polyethylene glycol (PEG); magnesium; topoisomerase; gyrase; accessory proteins; denaturating agents; cofactors; dNTPs; ATP; TTP; sequence-specific primer sets, including but not limited to unlabelled primers and labeled primers, such as biotinylated primers and LUX Primers; and random primers.

[0056] As used, the term “comprising” includes “consisting essentially of” and “consisting of”.

EXAMPLES

Example 1: Use of PYROPHAGE 3173 as a replacement for RT enzymes in RT-HDA

[0057] PYROPHAGE 3173 DNA polymerase has several advantages over Transcriptor and Thermoscript reverse transcriptase. For example, it is known that Thermoscript and Transcriptor have limited activity at 65°C in the HDA buffer. PYROPHAGE 3173 DNA

polymerase was tested to determine whether it could replace these reverse transcriptases in a one step isothermal RT-HDA amplification.

[0058] Briefly, 25 μL reaction mixtures were created comprising: (1) 0, 10, or 100 copies of an in vitro transcribed, synthetic RNA comprising the Chlamydia trachomatis cryptic plasmid RNA (ct-RNA) (GenBank Accession number X06707) (SEQ ID NO: 1); (2) forward primer 5'-ATC GCA TGC AAG ATA TCG AGT ATG CGT-3' (SEQ ID NO: 2) and reverse primer 5'-CTC ATA ATT AGC AAG CTG CCT CAG AAT-3' ("ct-*orf* primers") (SEQ ID NO: 3); (3) 2.5 U of Thermoscript, Thermo-X, Transcriptor, or Pyrophage 3173; (4) 2U of Bst polymerase; and (5) 1 U of *uvrD* helicase. Amplications were performed at 65°C for 75 minutes. Reaction mixtures contained the final concentrations of reagents set forth in Table 1. As can be seen at Fig. 1, PYROPHAGE 3173 performs as well as amplification other RT enzymes.

Reagent	Final Concentration
Tris-HCl	20 mM
KCl	10 mM
$MgSO_4$	4 mM
NaCl	40 mM
dNTP	0.4 mM
dATP	3 mM

Table 1

Example 2: Use of PYROPHAGE 3173 as a replacement for both RT and DNA-dependant DNA polymerase

[0059] PYROPHAGE 3173 DNA polymerase was tested to determine whether it could replace both reverse transcriptase and DNA-dependant DNA polymerase in a one step isothermal RT-HDA amplification. Briefly, 25 μL reaction mixtures were created comprising: (1) 0, 25, or 100 copies of a ct-RNA; (2) ct-*orf* primers; (3) 2.5 U of Pyrophage 3173; (4) either 0U or 2U of Bst polymerase; and (5) 1 U of *uvrD* helicase. Reaction mixtures contained the final concentrations of reagents set forth in Table 1. Amplications were performed at 62°C or 65°C for 75 minutes. Results are shown at Fig. 2. As can be seen, PYROPHAGE 3173 is capable of replacing both reverse transcriptase and DNA-dependant DNA polymerase in a one step isothermal RT-HDA.

[0060] PYROPHAGE 3173 DNA polymerase also was compared to other enzymes having reverse transcriptase activity for the ability to perform RT-HDA in the absence of a separate DNA-dependant DNA polymerase. Briefly, 25 μL reaction mixtures were created comprising: (1) 0, 25, or 100 copies of a ct-RNA; (2) ct-*orf* primers; (3) 2.5 U of Thermoscript, Thermo-X, Transcriptor, or Pyrophage 3173; and (4) 1 U of *uvrD* helicase. Amplifications were performed at 65°C for 75 minutes. Detection by Luminex as in Example 1. Results are shown at Fig. 3. In contrast to PYROPHAGE 3173, other reverse transcriptases are not effective substitutes for Bst-polymerase for RT-HDA. Little or no assay signal was observed for RT-HDA reactions utilizing Thermo-X, Thermoscript or Transcriptor, when Bst-polymerase was omitted. Only PYROPHAGE 3173 was able to generate signal in reaction which had no Bst-polymerase.

Example 3: Use of PYROPHAGE 3173 for DNA amplification

[0061] *Target amplification*. HPV16 DNA was used as the target DNA in an HDA assay. The double stranded DNA target was denatured in 5 μl 0.1M NaOH at 65°C for 10 minutes. An equal volume of 0.2M Hepes was then added to neutralize the denatured target. 15 μl of premix and 25 μl of amplification mix were added to the target and incubated at 65°C for 1.5 hours. Premix and amplification mix constituents are set forth in Table 2.

[0062] *Amplicon detection*. The HDA product (5 μL) was transferred to a U-bottom hybridization plate and then diluted in 5 μl of 1X denaturation reagent (Digene HC2 DNR, Qiagen Gaithersburg, Inc., Gaithersburg, MD). The plate then was sealed and shaken for 30 seconds at 1100 rpm in a Digene shaker and incubated at room temperature for 15 minutes. A hybridization diluent (5 μl of 1X hc2 probe diluent, Qiagen Gaithersburg, Inc., Gaithersburg, MD) was added and the plate was resealed and shaken for 30 seconds at 1100 rpm in a Digene shaker. A Luminex Bead Cocktail (10 μl in 1X TE) having an oligonucleotide complementary to the amplicon was added to each well (3000 beads/well), the plate was sealed again and incubated at 50°C for 30 minutes with shaking in the dark. Strepavidin-Phycoerythrin (Moss Corp.) (10 μl diluted to 12.5ng/μl in PBS) was added and the plate again sealed and shaken for 5 minutes at 1100 rpm in a Digene shaker protected from light. Phosphate buffered saline (150 μl) was then added, the plate resealed and shaken for 1 minute at 800 rpm. Median fluorescence intensity (MFI) was determined using a Luminex 100 and Luminex 1.7 software. Results are shown at Fig. 4. MFI above a background level correlates with presence of the DNA target.

Example 4: Detection of two HPV 16 mRNA sequences using one step RT-HDA

[0063] Synthetic, *in vitro* transcribed RNAs corresponding to the HPV 16 E6-7 gene and the HPV 16 L1 gene were used as targets. Either 25 or 250 copies of each target nucleic acid were included in each reaction. A one step isothermal RT-HDA reaction was run as in Example 2, using the primers set forth in Table 3 in place of the Ct-*orf* primers. The reverse primer was used at a final concentration of 75 mM, while the forward primer concentration was 35 mM, 40 mM, 45 mM, 50 mM, or 55 mM. Results are shown at Fig. 5. Detection of 25 copies each of the two HPV 16 RNAs was robust for the RT-HDA reactions. The optimal primer concentrations in this experiment was 75 mM each reverse, biotinylated primer and 40 or 45 mM of each forward primer. The coefficient of variation (n=3) for the S/N was low (12 to 22%) for these reactions.

	Reagents	Final Concentration	
Premix	10X Annealing Buffer (100 mM KCl and 200 mM Tris HCl pH 8.8)	1	X
	Forward Primer	50	nM
	Reverse Primer	75	nM
Amplification Mix	Reagents	Final Concentration	
	10X Annealing Buffer (100 mM KCl and 200 mM Tris HCl pH 8.8)	1	X
	$MgSO_4$	4	mM
	NaCl	40	mM
	dNTP	0.4	mM
	dATP	3	mM
	DNA polymerase (Bst/Pyrophage)	(0.4/0.1)	U/μl
	Tte-UvrD helicase	0.02	U/μl

Table 2

HPV16-L1	Forward primer SEQ ID NO: 4	5’ TGC CTC CTG TCC CAG TAT CTA AGG TT 3’
	Reverse primer SEQ ID NO: 5	5’ Biotin-TGC AAG TAG TCT GGA TGT TCC TGC 3’
HPV16-E	Forward primer SEQ ID NO: 6	5’ GCA ACC AGA GAC AAC TGA TCT CTA CTG 3’
	Reverse primer SEQ ID NO: 7	5’ Biotin-TTC TGC TTG TCC AGC TGG ACC ATC TA 3’

Table 3

Example 5: Use of PYROPHAGE 3173 in hybrid capture

[0064] ***A. Hybrid capture technology***

[0065] Hybrid capture technology utilizes certain antibodies capable of bind to RNA:DNA hybrids in various methods of purifying and detecting specific target nucleic acids in a sample. Various iterations of the hybrid capture method are described in, *inter alia*, U.S. Patent Nos. 5,994,079, 6,027,897, 6,277,579, 6,686,151, and 7,439,016; US Patent Publication Nos. 2006/0051809 A1, 2009/0162851 A1, and 2009-0298187 A1; and PCT Publication No. WO 01/96608, each of which is incorporated herein by reference in its entirety. The basic hybrid capture protocol comprises: (1) hybridizing a nucleic acid probe to the target nucleic acid to generate a DNA:RNA hybrid; (2) associating the DNA:RNA hybrid with a solid phase to facilitate isolation of the target nucleic acid; and (3) detecting the DNA:RNA hybrid. In various iterations, anti-DNA:RNA hybrid antibodies can be used in either step (2) or step (3). By way of example and not limitation, the anti-DNA:RNA hybrid antibody may be bound to the solid phase (covalently or otherwise), thereby mediating “capture” of the DNA:RNA hybrid to the solid phase. Alternatively, a nucleic acid probe bound to the solid phase (covalently or otherwise) may capture the DNA:RNA hybrid to the solid phase, which may then be detected by a detectably labeled anti-DNA:RNA hybrid antibody.

[0066] ***B. Detection of a target isolated via hybrid capture***

[0067] As noted previously, hybrid capture utilizes DNA:RNA hybrids. Therefore, the identity of the desired target will be important. When the target nucleic acid molecule is DNA,

the probe is preferably RNA and when the target nucleic acid is RNA, the probe is preferably DNA.

[0068] Sample comprising the target nucleic acid is collected in a tube and treated with a denaturation reagent, such as an alkaline solution, to render the target nucleic acid molecule accessible to hybridization. Additionally, alkaline treatment of protein effectively homogenizes the specimen to ensure reproducibility of analysis results for a given sample. It can also reduce the viscosity of the sample to increase kinetics, homogenize the sample, and reduce background by destroying any endogenous single stranded RNA nucleic acids, DNA-RNA hybrids or RNA-RNA hybrids in the sample. It also helps inactivate enzymes such as RNases and DNases that may be present in the sample. One skilled in that art would appreciate that if RNA is the target nucleic acid (as opposed to DNA), different reagents may be preferable including, but not limited to phenol extraction and TCA/acetone precipitation, and guanidinium thiocyanate-phenol-chloroform extraction.

[0069] After the sample containing the nucleic acid is denatured, it is contacted with one or more polynucleotide probes under a condition sufficient for the one or more polynucleotide probes to hybridize to the target nucleic acid in the sample to form a double-stranded nucleic acid hybrid. The probe can be full length, truncated, or synthetic DNA or full length, truncated, or synthetic RNA. If the target nucleic acid is DNA, then the probe may be RNA and if the target nucleic acid is RNA, then the probe may be DNA. Preferably, the one or more polynucleotide probes are diluted in a probe diluent that also can act as a neutralizing hybridization buffer (to neutralize the basic denaturation reagent). The probe diluent used for DNA or RNA probes will differ due to the different requirements necessary for DNA versus RNA stability. For example, if the probes are RNA, it is preferable to neutralize the sample first and than add the probe or alternatively, add the RNA probe and neutralizing agent (probe diluent) to the sample at the same time as NaOH can destroy RNA. The probe diluent can be used to dissolve and dilute the probe and also help restore the sample to about a neutral pH, e.g., about pH 6 to about pH 9, to provide a more favorable environment for hybridization. Sufficient volume of probe diluent, preferably one-half volume of the sample, may be used to neutralize the base-treated sample.

[0002] After the probes are allowed to hybridize to the target nucleic acid molecule and to form a double-stranded nucleic acid hybrid, the hybrid is captured by an anti-hybrid antibody

that is immobilized onto a paramagnetic beads. The hybrids are incubated with the anti-hybrid antibody at about 67ºC to about 70ºC for about 30 minutes. A magnetic field is then applied to the tube and the supernatant removed from the beads. The beads may then be washed with a suitable wash buffer comprising, for example, 40 mM Tris, pH 8.2, 100 mM NaCl, 0.5% Triton-X 100 and 0.05% sodium azide.

[0070] The captured nucleic acids may then be detected using any of the amplification schemes described herein.

[0071] ***C. Increasing sensitivity of a hybrid capture assay***

[0072] In some iterations, amplification can be used to increase the sensitivity of hybrid capture assays, particularly when the target nucleic acid is expected to be present in low copy numbers. However, standard amplification techniques are not always compatible with the conditions in which hybrid capture may be used. For example, one particular application of hybrid capture technology is for screening assays in rural communities, where expensive thermocyclers and trained technicians are often unavailable. In such circumstances, it is beneficial to reduce the number of complicated reagents involved and simplify the steps, which often precludes use of standard PCR protocols. In such a circumstance, thermostable polymerases such as PYROPHAGE 3173 would be useful.

[0073] In one example, an amplification as described herein may be performed on a sample, with the resultant amplicons purified and detected by a hybrid capture assay as set forth in, for example, U.S. Patent Nos. 5,994,079, 6,027,897, 6,277,579, 6,686,151, and 7,439,016; US Patent Publication Nos. 2006/0051809 A1, 2009/0162851 A1, and 2009-0298187 A1; and PCT Publication No. WO 01/96608.

[0074] Alternatively, the target nucleic acid may be purified as set forth in Example 5B, then amplified as set forth herein. After separation, targets may be denatured, separated from the beads, and detected by a hybrid capture assay as set forth in, for example, U.S. Patent Nos. 5,994,079, 6,027,897, 6,277,579, 6,686,151, and 7,439,016; US Patent Publication Nos. 2006/0051809 A1, 2009/0162851 A1, and 2009-0298187 A1; and PCT Publication No. WO 01/96608.

[0075] ***D. Adapting incompatible targets for use with available reagents***

[0076] As set forth above, hybrid capture is preferably used in combination with DNA:RNA hybrids. However, there may be instances where the target nucleic acid and the

hybrid capture probes are both RNA. In such a case, it would be desirable to convert the target RNA to a DNA before performing hybrid capture. The reverse transcriptase activity of PYROPHAGE 3173 could be useful in such an embodiment.

[0077] RNA optionally may be extracted from the sample before performing the reverse transcription reaction, particularly if the desired target has a DNA equivalent likely to be present in the sample, such as when an mRNA is the desired target. Many methods of isolating total RNA and subsets thereof are well known in the art, including, for example, acid guanidinium thiocyanate-phenol-chloroform extraction and commercially available kits, such as the RNeasy® line of kits (Qiagen GmbH, Hilden, DE). Whether to isolate RNA before performing the reverse transcription reaction and the precise method of doing so will depend largely on the particular target and application and can be determined by a person of ordinary skill in the art.

[0078] Once the sample is prepared as desired, the reverse transcription reaction can be performed essentially as described herein. Where increased sensitivity is necessary or desired, a one step RT-HDA reaction may likewise be performed. Target isolation may then be performed by hybrid capture as described in Example 5B and/or target detection may be performed as described in, for example, U.S. Patent Nos. 5,994,079, 6,027,897, 6,277,579, 6,686,151, and 7,439,016; US Patent Publication Nos. 2006/0051809 A1, 2009/0162851 A1, and 2009-0298187 A1; and PCT Publication No. WO 01/96608.

What is claimed is:

1. A method for isothermal amplification of a target nucleic acid, the method comprising reacting the target nucleic acid with a reaction mixture comprising:

   a) a first enzyme having a helicase activity; and
   b) a second enzyme having:
      i. a reverse transcriptase activity; and
      ii. a DNA-dependant DNA polymerase activity.

2. The method of claim 1, wherein the target nucleic acid is selected from the group consisting of dsDNA, dsRNA, ssDNA, or ssRNA.

3. The method of claim 1 or claim 2, wherein the second enzyme with reverse transcriptase activity is PYROPHAGE 3173.

4. The method of any of claims 1–3 wherein the target nucleic acid is an HPV nucleic acid.

5. The method of any of claims 1–4 wherein the reaction mixture further comprises a target specific nucleic acid primer.

6. The method of any of claims 1–4 wherein the reaction mixture comprises a random primer.

7. The method of any of claims 1–6 wherein the reaction mixture further comprises a topoisomerase or a gyrase.

8. The method of any of claims 1–7 wherein the target nucleic acid is a target RNA and said second enzyme converts the target RNA to a target DNA by a method comprising a reverse transcription reaction.

9. The method of claim 8 further comprising an amplification reaction wherein the second enzyme amplifies the target DNA.

10. The method of any of claims 1–9 wherein the reaction mixture comprises:

   a. KCl;
   b. Tris HCl;

c. $MgSO_4$;

d. NaCl;

e. dNTP;

f. dATP; and

g. a primer set

11. The method of claim 10 wherein said primer set is selected from the group consisting of a target specific nucleic acid primer set and a random primer set.

12. The method of any of claims 1–11 wherein said primer set comprises a primer selected from the group consisting of SEQ ID NO: 2 through SEQ ID NO: 7.

13. The method of any of claims 1–12 further comprising isolating the target nucleic acid from a sample.

14. The method of claim 13 wherein the target nucleic acid is isolated from the sample by a method comprising:

a. generating a DNA:RNA hybrid comprising the target nucleic acid;

b. binding the DNA:RNA hybrid to a solid phase; and

c. separating the DNA:RNA hybrid bound to the solid phase from the sample.

15. The method of claim 14 wherein the DNA:RNA hybrid is bound to an anti-DNA:RNA antibody.

16. The method of claim 15 wherein the anti-DNA:RNA antibody is bound or adapted to be bound to the solid phase.

17. The method of claim 13 wherein the target nucleic acid is purified from the sample before the target nucleic acid is amplified.

18. The method of claim 13 wherein the target nucleic acid is purified from the sample after the target nucleic acid is amplified.

19. A kit comprising

a) a first enzyme having a helicase activity; and

b) a second enzyme having:

i. a reverse transcriptase activity; and

ii. a DNA-dependant DNA polymerase activity.

20. The kit of claim 19, further comprising at least one component selected from the group consisting of:

a. KCl;

b. Tris HCl;

c. $MgSO_4$;

d. NaCl;

e. dNTP;

f. dATP;

g. a gyrase

h. a topoisomerase;

i. a primer set;

j. a nucleic acid probe;

k. an anti-DNA:RNA hybrid antibody; and

l. a solid phase,

wherein each component optionally is a component of a stock solution.

21. The kit of claim 19 or claim 20 comprising a nucleic acid probe, an anti-DNA:RNA hybrid antibody, and a solid phase, wherein either:

a. the anti-DNA:RNA antibody is adapted to be bound to the solid phase or is bound to the solid phase; or

b. the nucleic acid probe is adapted to be bound to the solid phase or is bound to the solid phase.

22. The kit of any of claims 19–21 comprising an stock annealing buffer comprising KCl and Tris HCl.

23. The kit of claim 22 wherein said annealing buffer is formulated so as to be dilutable to a final concentration of 10 mM KCl and 20mM Tris-HCl.

24. The kit of any of claims 19–23 wherein the second enzyme is PYROPHAGE 3173.

25. A mixture comprising:

a. a target nucleic acid;

b. a first enzyme having a helicase activity; and

c. a second enzyme having:

i. a reverse transcriptase activity; and

ii. a DNA-dependant DNA polymerase activity.

26. The mixture of claim 25, further comprising at least one component selected from the group consisting of:

a. KCl;

b. Tris HCl;

c. $MgSO_4$;

d. NaCl;

e. dNTP;

f. dATP;

g. a gyrase

h. a topoisomerase;

i. a primer set;

j. a nucleic acid probe;

k. an anti-DNA:RNA hybrid antibody; and

l. a solid phase.

27. The mixture of claim 25 or 26 comprising a nucleic acid probe, an anti-DNA:RNA hybrid antibody, and a solid phase, wherein either:

a. the anti-DNA:RNA antibody is adapted to be bound to the solid phase or is bound to the solid phase; or

b. the nucleic acid probe is adapted to be bound to the solid phase or is bound to the solid phase.

28. The mixture of any of claims 25–27 comprising the following components in aqueous solution:

a. 10mM KCl;

b. 20mM Tris HCl;

c. 4 mM $MgSO_4$;

d. 40 mM NaCl;

e. 0.4 mM dNTP;

f. 3 mM dATP.

29. The mixture of claim any of claims 25–28 wherein the second enzyme is PYROPHAGE 3173.

30. An amplified nucleic acid obtained by the method of any of claims 1–18.

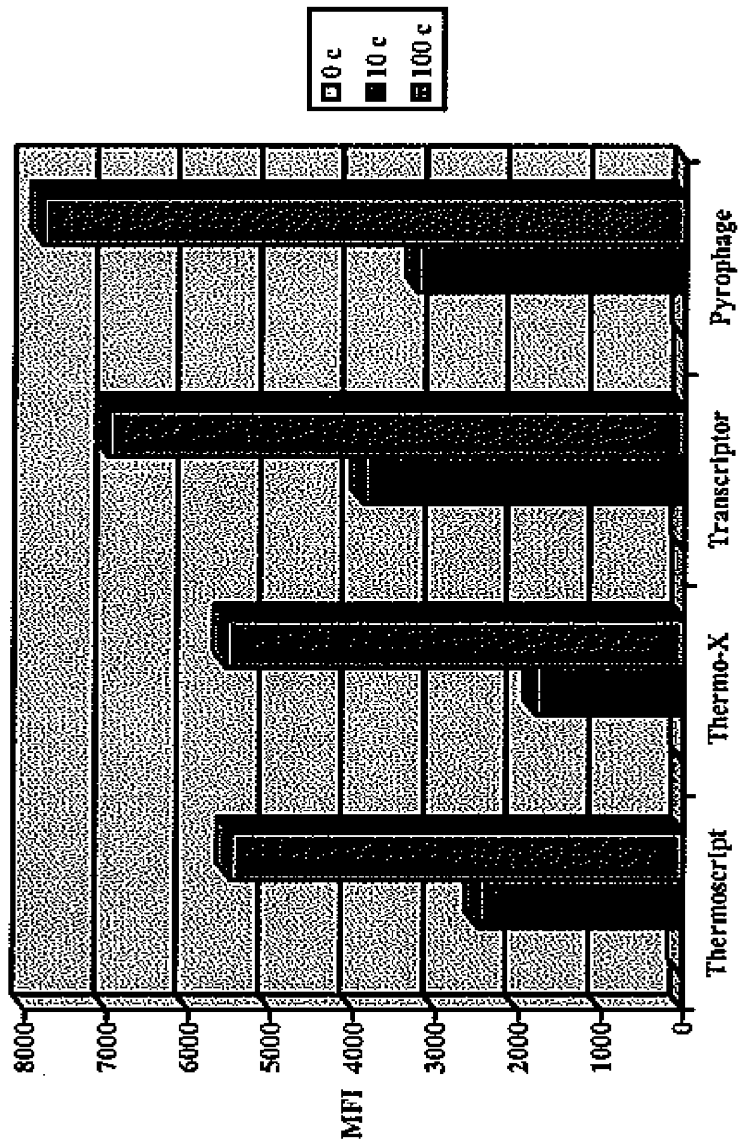

Fig. 1

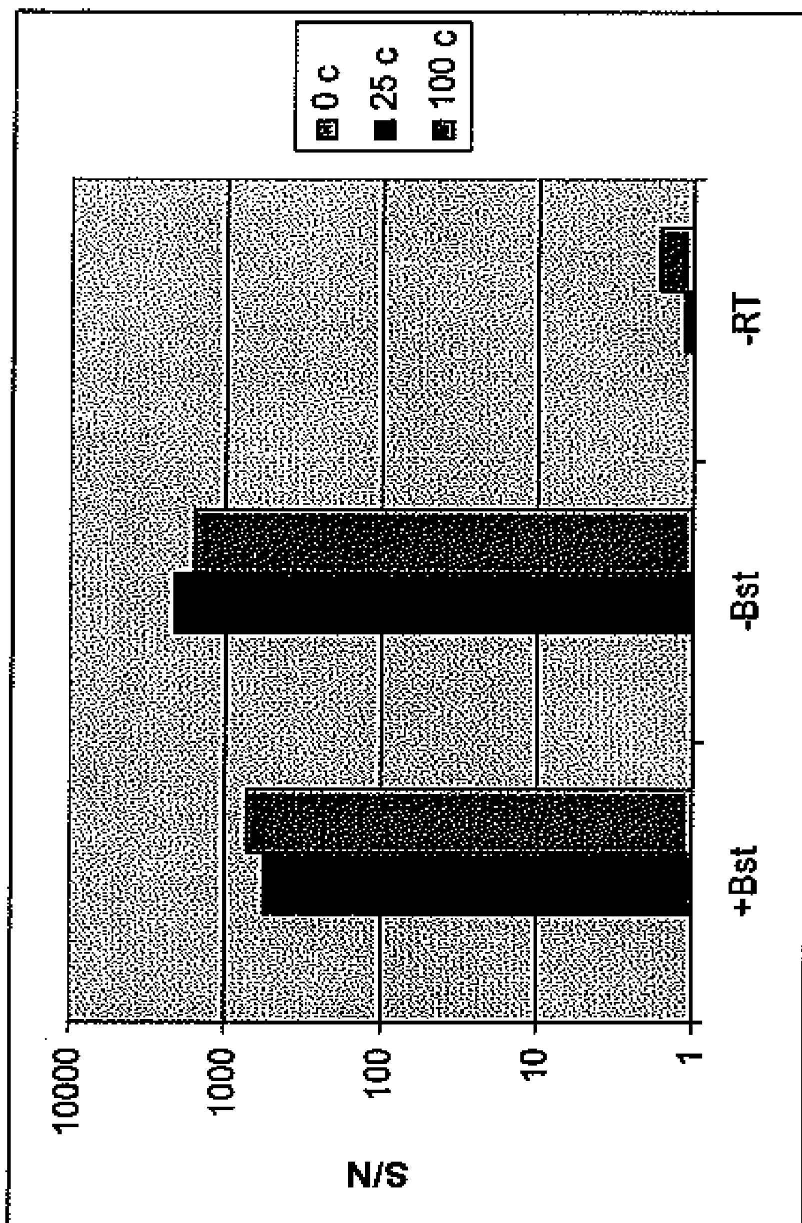

Fig. 2

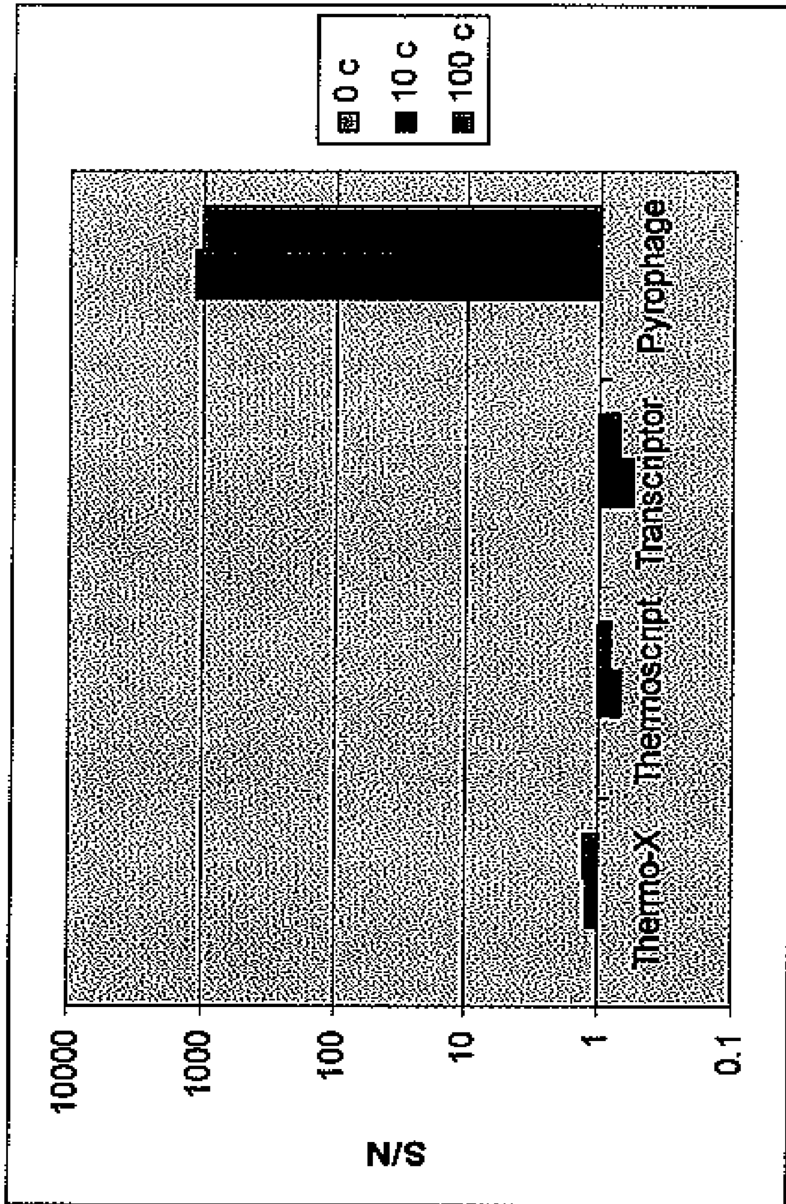

Fig. 3

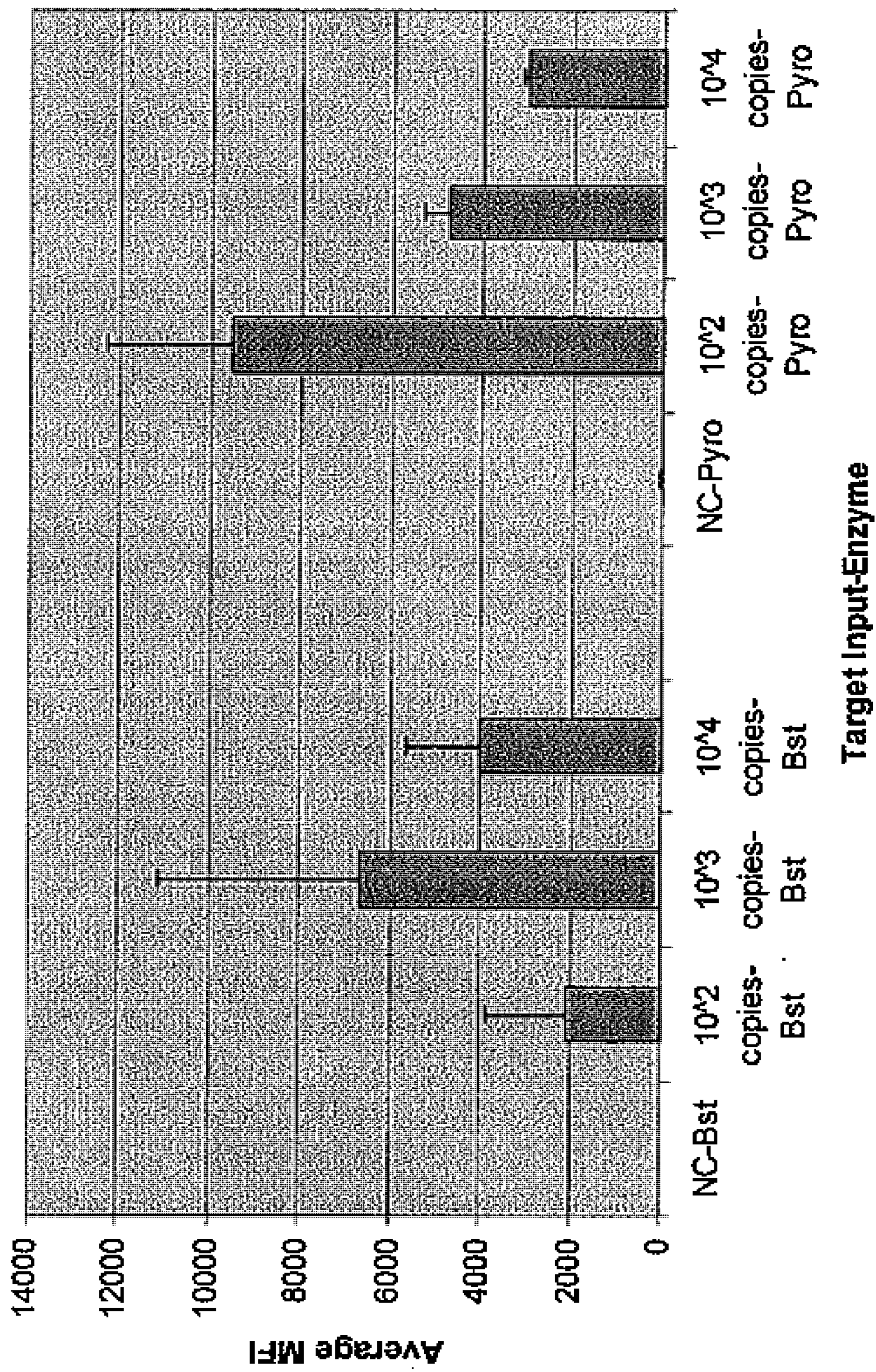

Fig. 4

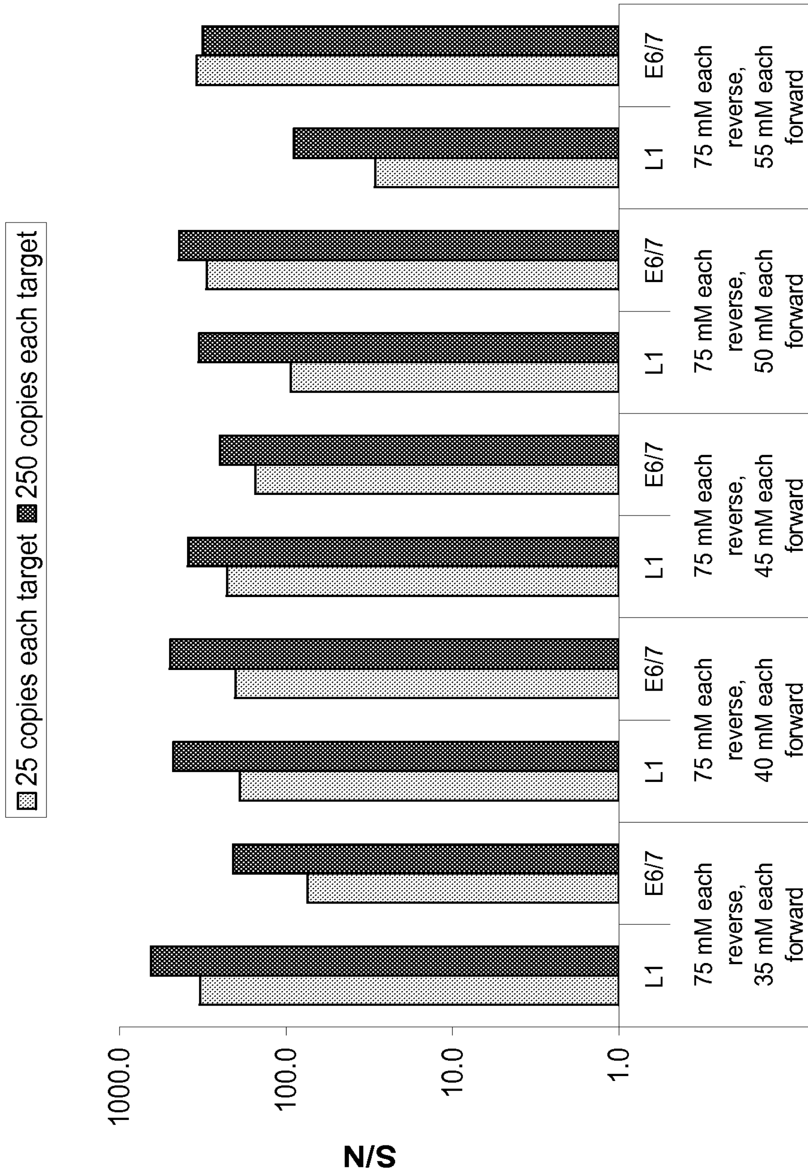

Fig. 5

INTERNATIONAL SEARCH REPORT

International application No
PCT/US2011/020459

A. CLASSIFICATION OF SUBJECT MATTER
INV. C12Q1/68
ADD. C12N9/12 C12N9/90

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
C12Q C12N

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

EPO-Internal, WPI Data, BIOSIS, CAB Data, Sequence Search

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X,P	WO 2010/088273 A1 (QIAGEN GAITHERSBURG [US]; DOSEEVA VICTORIA [US]; FORBES THOMAS [US]; W) 5 August 2010 (2010-08-05) page 33, line 13 - page 34, line 8	1,2,4-6, 19-22, 25-27,30
X	WO 2006/074334 A2 (BIOHELIX CORP [US]; KONG HUIMIN [US]; TANG WEN [US]) 13 July 2006 (2006-07-13) paragraph [0051]; claims 1-14	1,2,4-6, 19-22, 25-27,30
X	WO 2007/044671 A2 (LUCIGEN CORP [US]; SCHOENFELD THOMAS W [US]; DHODDA VINAY K [US]; DIFR) 19 April 2007 (2007-04-19) cited in the application claims 1-42	30

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[X] Further documents are listed in the continuation of Box C.

[X] See patent family annex.

* Special categories of cited documents :

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Date of the actual completion of the international search	Date of mailing of the international search report
15 March 2011	25/03/2011

Name and mailing address of the ISA/	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+31-70) 340-2040, Fax: (+31-70) 340-3016	Hornig, Horst

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C(Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	WO 2007/120808 A2 (BIOHELIX CORP [US]; TANG WEN [US]; KONG HUIMIN [US]) 25 October 2007 (2007-10-25) the whole document -----	30
X	WO 2005/095654 A1 (BIOHELIX CORP [US]; KONG HUIMIN [US]; XU YAN [US]) 13 October 2005 (2005-10-13) the whole document -----	30
X	WO 2004/027025 A2 (NEW ENGLAND BIOLABS INC [US]) 1 April 2004 (2004-04-01) the whole document -----	30
X	MOTRE A ET AL: "Enhancing helicase-dependent amplification by fusing the helicase with the DNA polymerase", GENE, ELSEVIER, AMSTERDAM, NL, vol. 420, no. 1, 15 August 2008 (2008-08-15), pages 17-22, XP022820485, ISSN: 0378-1119, DOI: DOI:10.1016/J.GENE.2008.04.017 [retrieved on 2008-05-08] the whole document -----	30
X	GOLDMEYER JAMES ET AL: "Development of a novel one-tube isothermal reverse transcription thermophilic helicase-dependent amplification platform for rapid RNA detection.", THE JOURNAL OF MOLECULAR DIAGNOSTICS : JMD NOV 2007 LNKD- PUBMED:17975029, vol. 9, no. 5, November 2007 (2007-11), pages 639-644, XP002628183, ISSN: 1525-1578 the whole document -----	30
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INTERNATIONAL SEARCH REPORT

International application No
PCT/US2011/020459

C(Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	AN LIXIN ET AL: "Characterization of a thermostable UvrD helicase and its participation in helicase-dependent amplification", JOURNAL OF BIOLOGICAL CHEMISTRY, AMERICAN SOCIETY FOR BIOCHEMISTRY AND MOLECULAR BIOLOGY, INC, US, vol. 280, no. 32, 13 June 2005 (2005-06-13), pages 28952-28958, XP002400121, ISSN: 0021-9258, DOI: DOI:10.1074/JBC.M503096200 the whole document -----	30

Form PCT/ISA/210 (continuation of second sheet) (April 2005)

INTERNATIONAL SEARCH REPORT

Information on patent family members

International application No
PCT/US2011/020459

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
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WO 2006074334 A2	13-07-2006	EP 1833995 A2	19-09-2007
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		JP 2009511019 T	19-03-2009
		US 2008268498 A1	30-10-2008
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		JP 2006500028 T	05-01-2006

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(19)

(11) **EP 2 824 189 A1**

(12) **EUROPEAN PATENT APPLICATION**
published in accordance with Art. 153(4) EPC

(43) Date of publication:
14.01.2015 Bulletin 2015/03

(21) Application number: **13757504.9**

(22) Date of filing: **11.03.2013**

(51) Int Cl.:
C12Q 1/42 $^{(2006.01)}$ ***C12Q 1/68*** $^{(2006.01)}$
C12Q 1/48 $^{(2006.01)}$

(86) International application number:
PCT/KR2013/001935

(87) International publication number:
WO 2013/133680 (12.09.2013 Gazette 2013/37)

(84) Designated Contracting States:
AL AT BE BG CH CY CZ DE DK EE ES FI FR GB GR HR HU IE IS IT LI LT LU LV MC MK MT NL NO PL PT RO RS SE SI SK SM TR
Designated Extension States:
BA ME

(30) Priority: **09.03.2012 KR 20120024676**

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(54) **COMPOSITION FOR HOT-START REVERSE TRANSCRIPTION REACTION OR HOT-START REVERSE TRANSCRIPTION POLYMERASE CHAIN REACTION**

(57) The present invention relates to a composition for hot-start reverse transcription reaction and a composition for reverse transcription PCR, which comprises the same, and more particularly to a reaction composition obtained by adding pyrophosphate and pyrophosphatase to an aqueous solution containing reaction buffer solution, $MgCl_2$, four kinds of dNTPs, and reverse transcription polymerase in a single reaction tube, and a composition for hot-start reverse transcription reaction obtained by freezing or drying the reaction composition and having increased stability and long- term storage stability. Also, the present invention relates to a composition that comprises DNA polymerase in the composition for hot-start reverse transcription reaction, and thus enables a hot-start reverse transcription reaction and a PCR reaction to be sequentially performed, and to a method for amplifying nucleic acid the same. In comparison with conventional compositions for reverse transcription reaction, the composition for hot-start reverse transcription reaction can prevent non-specific reverse transcription reactions during mixing at room temperature and can selectively perform the reverse transcription of the target RNA at the reaction temperature, making it possible to amplify the reverse transcription product by PCR with high sensitivity. Thus, the composition of the invention can be conveniently and effectively used in multiplex reverse transcription PCRs or real-time quantitative reverse transcription PCR.

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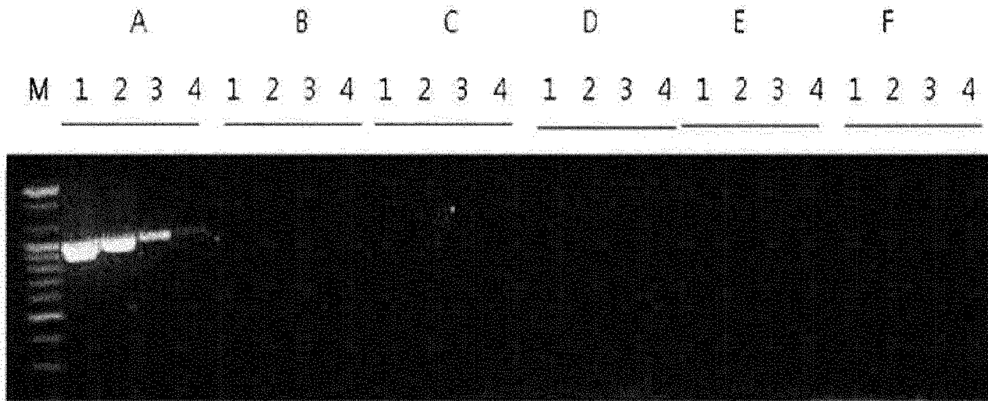
Fig. 1



Description

TECHNICAL FIELD

[0001] The present invention relates to a composition for hot-start reverse transcription reaction or hot-start reverse transcription polymerase chain reaction.

BACKGROUND ART

[0002] Reverse transcription reactions are classified into reactions employing random primers, and reactions employing target-specific primers. In reverse transcription reactions for detection of a specific target, such as diagnostic kits for detection of RNA virus or the like, target-specific primers are generally used because they show higher sensitivity. In such reverse transcription reactions, specificity and sensitivity are determined by the high selectivity of primers that bind specifically to a target RNA sequence. However, because all components required for a reverse transcription reaction are mixed at room temperature, a non-specific reverse transcription reaction is caused by non-specific priming under this condition. Even at room temperature, non-specific priming occurs due to the high activity of reverse transcriptase to produce a number of unspecific cDNAs, and thus it is a major factor that increases non-specific amplification reactions. Due to such non-specific reverse transcription reactions, primers required for subsequent PCR reactions and limited concentrations of other essential components are consumed, and for this reason, the non-specific reverse transcription reactions act as competitive inhibitors. Non-specific reverse chain reactions are problematic when detecting a low concentration of RNA, and particularly, interfere with detection of a target RNA that is present in a very small amount in a solution that contains large amounts of RNAs extracted from cells or body fluids and having a high nucleotide sequence complexity. Thus, non-specific reverse transcription reactions make it difficult to detect a virus or gene present at a low concentration. Also, in multiplex reverse transcription reactions that are performed using various primers at the same time, non-specific reverse transcription reactions reduce specificity, making multiple detections difficult. This non-specific amplification is more greatly influenced by the relative amounts of the target nucleic acid and other nucleic acids derived from a biological sample than the absolute amount of the target nucleic acid. This is because non-specific hybridization of primers increases to increase non-specific reactions, when many RNAs other than a desired target are present in a reaction mixture.

[0003] In order to reduce such undesirable non-specific reactions and amplification, various attempts have been made. Specifically, there was an attempt to increase the detection limit of a specific target by more strongly hybridizing primers to the target. For example, a method was reported, in which the 5' terminus of primers are substituted with LNA so that it can more strongly hybridize to the target, thereby reducing non-specific amplification (Malgoyre A. et al., Biochem Biophys Res Commun. Mar 2, 2007; 354(1):246-52). As another method for solving problems using a specific primer structure, a method of preventing primer dimer formation was also developed. When a reverse transcription reaction is performed particularly at low temperatures, polymerization can proceed by partial hybridization between primers to form a dimer, an important factor that rapidly reduces sensitivity in the reverse transcription reaction. In order to solve this problem, it was proposed to use primers formed by extending complementary nucleotide sequences so that five nucleotides of the 5' nucleotide sequence of the primers can form a hairpin structure at low temperature (Ji Young Hong et al., Virol J. 2011; 8: 330. Published online 2011; Korean Patent No. 10-0987352). However, this lock primer method has disadvantages in that, because the primers have high hybridization temperature due to the nucleotide sequence added to the 5' terminus, non-specific hybridization of the primers to non-specific targets having nucleotide sequences similar thereto in a subsequent PCR reaction can be induced, and the efficiency of hybridization in the hybridization step is reduced due to the hairpin structure of the primers.

[0004] To solve such problems, a method was developed which uses blocked primers that are partially complementary to primers and that are blocked at the 3' terminus. Such blocked primers have advantages in that, because they are blocked at the 3' terminus, they do not act as primers in a nucleic acid polymerization reaction, and because they are short in length, they hybridize to primers only at room temperature to prevent primer dimer formation, and because they are detached immediately from the primers and are not operated when the temperature increases in a subsequent reaction, instant reverse transcription PCR can be performed (Korean Patent Application No. 10-2011-0017226).

[0005] In addition, a method of performing a reverse transcription reaction at high temperature for more specific hybridization to a target RNA was developed. It was reported that, when a cDNA is synthesized at high temperature such as 70°C using primers that hybridize at high temperature and a reverse transcription polymerase that operates even at high temperature, it can be more specifically amplified (Fuchs B et al., Mol Biotechnol, 1999 Oct;12(3):237-40).

[0006] In multiplex reverse transcription PCRs, specificity is more important. This is because several primers are used in the reaction, a non-specific reaction can occur in a subsequent PCR reaction due to a non-specific reaction in the reverse transcription reaction step. To avoid this non-specific reaction in multiplex reverse transcription PCRs, a reverse transcription reaction is first performed, and then a PCR reaction is performed, so that a more specific reaction can be

achieved. However, in this case, the process of opening a reaction tube and adding a reaction solution to the open reaction tube is troublesome, and the possibility of contamination in the process of opening and operating the reaction tube is higher. For this reason, it is generally preferable to sequentially perform a reverse transcription reaction and PCR in a single tube. Thus, a method of physically separating reactions from each other while performing reverse transcription PCR in a single tube without opening the tube was developed, in which the reverse transcription reaction mixture is present at the bottom of the tube during the reaction, and the PCR reaction mixture is suspended from the cover. In this method, the reverse transcription reaction is performed in the tube while the PCR reaction mixture is suspended from the cover and is not reacted, and after completion of the reverse transcription reaction, and the reaction tube is rotated in a centrifuge so that the PCR solution suspended from the cover of the reaction tube is mixed with the reverse transcription reaction product, after which the PCR reaction is performed (Rodney Mark Ratcliff, et al., 2002 November; 40(11): 40914099. doi: 10.1128/JCM.40.11.4091-4099.2002).

[0007] As a result of efforts to solve the above-described problems occurring in conventional reverse transcription reactions, a "hot-start reverse transcription reaction" was developed. The hot-start reverse transcription reaction is a method for detecting a very small amount of a target RNA, in which a reverse transcription reaction can be initiated at a high temperature at which priming could occur only to an RNA having a nucleotide sequence exactly complementary to primers, thereby preventing non-specific priming from occurring at room temperature and preventing non-specific primer oligomerization, thereby increasing the specificity of the reverse transcription reaction. To implement this method, a method that uses a heat-resistant polymerase and an aptamer was developed and is being used. A light cycler RNA master kit (Roche) is a method employing Tth DNA polymerase and an aptamer. Tth DNA polymerase combines a function of polymerizing DNA using RNA as a template and a function of polymerizing DNA using DNA as a template. The aptamer used herein is attached to the reactive site of Tth DNA polymerase, and thus is inactive at room temperature. When the temperature of the reaction solution is increased to high temperature, the three-dimensional structure of the aptamer is modified so that it is separated from Tth DNA polymerase so as to be active, and the reverse transcription reaction of a specifically primed target RNA can be performed, and afterwards PCR can be performed. In addition, the GeneAmp AccuRT Hot Start RNA PCR kit (Applied Biosystems) uses a heat-resistant polymerase derived from *Thermus* specie Z05 and can perform a reverse transcription reaction and PCR by using a single enzyme, like the Tth DNA polymerase. Also, the reaction is performed using an aptamer specific thereto. Such products can reduce non-specific amplification in reverse transcription PCR by inhibiting enzymatic activity at room temperature using the aptamer to reduce non-specific reverse transcription reactions. However, when the temperature of the aptamer is increased to high temperature, the three-dimensional structure thereof is modified, but when the temperature is lowered, the aptamer is restored to its original structure to inhibit DNA polymerases. In other words, the aptamer has the problem of reversibly inhibiting DNA polymerases. For the GeneAmp AccuRT Hot Start RNA PCR kit, it is described that the aptamer is still attached to DNA polymerase even at 55°C and is completely detached when the temperature becomes 65°C. Thus, in reactions in which the annealing temperature of primers that are generally used is 55°C or below, there is a problem in that the activity of DNA polymerase is inhibited to reduce PCR efficiency. For this reason, a heat-resistant DNA polymerase such as Tth DNA polymerase, which has reverse transcription function, is used. This heat-resistant DNA polymerase has a problem in that, because it has reverse transcription function during a subsequent PCR process in which enzymatic activity is maintained, it causes a continuous non-specific reverse transcription reaction, resulting in non-specific amplification.

[0008] A hot-start PCR reaction method employing antibodies is applicable only to Taq DNA polymerase (Enneth, G. et al., 1994, Biotechnology, 12; 506-509). In order to perform hot-start reverse transcription reactions using other kinds of reverse transcription polymerases, a heat-resistant reverse transcriptase that is resistant at high temperature should be used, and for reverse transcription PCR, a reverse transcriptase-specific antibody that is detached at low temperature and a DNA polymerase-specific antibody that is detached at high temperature are required. For this reason, antibodies specific to the respective heat-resistant polymerases should be developed, and thus there is a technical limit to such hot-start reverse transcription reactions.

[0009] The present inventors developed a hot-start PCR method that uses pyrophosphate (PPi) and heat-resistant pyrophosphatase (PPase) (Korean Patent No. 10-0292883). This method is based on the principle in which PPi that strongly binds to magnesium ions essential for DNA polymerization is added to inhibit a polymerase reaction at room temperature, and then PPase is reacted at high temperature to remove PPi. Reverse transcriptase cannot be used in a hot-start method that uses an antibody, because of its low activation temperature. A conventional hot-start PCR method that uses an antibody is a method in which the activity of enzyme is inhibited by enzyme-antibody binding at low temperature, and the antibody loses its binding with the enzyme due to a decrease in its stability when it reaches high temperature, whereby a PCR reaction occurs. Reverse transcriptase has no thermal stability at high temperature, unlike DNA polymerase. When the antibody reaches high temperature to lose its binding with reverse transcriptase, the activity of reverse transcriptase is inhibited. In an attempt to solve this problem, a hot-start reverse transcription reaction method that uses PPi and PPase has advantages in that it can be generally applied regardless of the kind of DNA polymerase and in that reactivity can be continuously maintained by continuously removing PPi generated from dNTP during PCR.

However, if a hot-start PCR master mix solution is made using this method, PPase will slowly dissolve PPi from Mg^{2+} ions so that the activity of DNA polymerase will appear, and ultimately, a desired hot-start PCR reaction effect will be lost. In addition, if a reverse transcription reaction master mix solution containing PPase is made, it has a problem in that the activity of PPase at room temperature or 4°C is maintained only for a short period of time, because PPase is very unstable. Thus, a mixture in a dried form having increased stability was developed (Korean Patent No. 10-1098764). Accordingly, in order to solve the above-described problems of hot-start reverse transcription reactions, technology for more highly sensitive hot-start reverse transcription and reverse transcription PCR is required.

DISCLOSURE OF INVENTION

[0010] It is an object of the present invention to provide a composition for hot-start reverse transcription reaction or hot-start reverse transcription PCR, which comprises pyrophosphate (PPi) and pyrophosphatase (PPase).

[0011] To achieve the above object, the present invention provides a composition for hot-start reverse transcription reaction, which comprises an Mg^{2+} ion, four kinds of dNTPs, reverse transcription polymerase, pyrophosphate (PPi), and pyrophosphatase (PPase).

[0012] The present invention also provides a composition for hot-start reverse transcription PCR, which comprises an Mg^{2+} ion, four kinds of dNTPs, reverse transcription polymerase, pyrophosphate (PPi), and pyrophosphatase (PPase), and further comprises DNA polymerase.

[0013] The present invention also provides a method of preparing a composition for a hot-start reverse transcription reaction, the method comprising introducing the above-described composition for the hot-start reverse transcription reaction into a single reaction tube.

[0014] The present invention also provides a kit for hot-start reverse transcription reaction, which comprises the above-described composition for hot-start reverse transcription reaction.

[0015] The present invention also provides a method for amplifying nucleic acid, the method comprising the steps of: mixing the above-described composition for hot-start reverse transcription PCR with a sample containing template RNA to obtain a reaction mixture; performing a reaction so as to amplify the reaction mixture to thereby obtain an amplification product; and analyzing the amplification product.

BRIEF DESCRIPTION OF THE DRAWINGS

[0016]

FIG. 1 shows the effect of pyrophosphate (PPi) on the inhibition of reverse transcription reactions:

- Lanes 1, 2, 3 and 4: the results of performing reverse transcription reactions of 100 ng, 10 ng, 1 ng and 100 pg of RNA, respectively;
- A: positive control results obtained by performing a reverse transcription reaction in the absence of PPi, followed by PCR reaction;
- B, C, D, E and F: the results of performing reverse transcription reactions in the presence of varying concentrations of PPi, followed by PCR reactions; and
- Lane M: 100-bp DNA ladder (Bioneer, Korea) for sizing of PCR products.

FIG. 2 shows that reverse transcription reactions are activated again by adding pyrophosphatase (PPase) that hydrolyzes PPi into two phosphates:

- Lanes 1, 2, 3 and 4: the results of performing reverse transcription reactions of 100 ng, 10 ng, 1 ng and 100 pg of RNA, respectively;
- A: positive control results obtained by performing a reverse transcription reaction in the absence of PPi, followed by PCR reaction;
- B, C, D, E and F: the results obtained by adding varying concentrations of PPi;
- B-1, C-1, D-1, E-1 and F-1: the results of performing reverse transcription reactions in the presence of PPase, followed by PCR reactions;
- Lane M: 1-kb DNA ladder.

FIG. 3 shows the effects of addition of PPi and PPase on real-time reverse transcription PCR:

- Lanes 1, 2, 3 and 4: the results of real-time reverse transcription PCR using 100 ng, 10 ng, 1 ng and 100 pg of human total RNA.

FIG. 4 shows the results of detecting hepatitis C virus RNA by a hot-start reverse transcription reaction.
FIG. 5a shows the effects of PPi and PPase on the inhibition of non-specific reactions in real-time reverse transcription PCR:

A: the results obtained using a reaction solution having no hot-start reverse transcription function;
B: the results obtained from adding 1 μg of total RNA of human cell to the reaction solution of "A";
C: the results obtained using a hot-start reverse transcription reaction solution comprising PPi and PPase; and
D: the results obtained from adding 1 μg of total RNA of human cell to the reaction solution of "C".

FIG. 5b shows the results of electrophoresis of real-time reverse transcription PCR products on 2% agarose gel:

Lanes 1, 2, 3, 4, 5 and 6: products obtained from template hepatitis C virus RNAs having copy numbers of 10^6, 10^5, 10^4, 10^3, 10^2 and 10;
A: the results obtained using a reaction solution having no hot-start reverse transcription function;
B: the results obtained from adding 1 μg of total RNA of human cell to the reaction solution of "A";
C: the results obtained using a hot-start reverse transcription reaction solution comprising PPi and PPase; and
D: the results obtained from adding 1 μg of total RNA of human cell to the reaction solution of "C".

FIG. 6a shows a comparison of the inhibition of non-specific reactions in real-time reverse transcription PCR between the use of hot-start reverse transcription reaction solution and Taq antibody:

A: the results obtained using a reaction solution having no hot-start reverse transcription function, which comprises Taq antibody, together with a hot-start PCR reaction solution;
B: the results obtained from adding 1 μg of total RNA of human cell to the reaction solution of "A";
C: the results obtained using a hot-start reverse transcription reaction solution, which comprises PPi and PPase, together with a hot-start PCR reaction solution; and
D: the results obtained from adding 1 μg of total RNA of human cell to the reaction solution of "C";

FIG. 6b shows the results of electrophoresis of real-time reverse transcription PCR products on 2% agarose gel:

Lanes 1, 2, 3, 4, 5 and 6: products obtained from template hepatitis C virus RNAs having copy numbers of 10^6, 10^5, 10^4, 10^3, 10^2 and 10;
A: the results obtained using a reaction solution having no hot-start reverse transcription function, which comprises Taq antibody, together with a hot-start PCR reaction solution;
B: the results obtained from adding 1 μg of total RNA of human cell to the reaction solution of "A";
C: the results obtained using a hot-start reverse transcription reaction solution, which comprises PPi and PPase, together with a hot-start PCR reaction solution;
D: the results obtained from adding 1 μg of total RNA of human cell to the reaction solution of "C";

FIGS. 7a and 7c are the graphs of real-time reverse transcription PCR, which illustrate that a solution-state reverse transcription/PCR composition and a dry-state reverse transcription/PCR composition exhibit equal performance in real-time reverse transcription PCR (FIG. 7a: solid state; FIG. 7c: dry state).
FIGS. 7b and 7d are the standard graphs of real-time reverse transcription PCR, which illustrate that a solution-state reverse transcription/PCR composition and a dry-state reverse transcription/PCR composition exhibit equal performance in real-time reverse transcription PCR (FIG. 7b: solid state; FIG. 7d: dry state).
FIG. 8a is a graphic diagram showing the results of performing real-time reverse transcription PCR using a liquid-state mixture as a control in order to test the stability of a dry reverse transcription/PCR composition as a function of storage period.
FIG. 8b is a graphic diagram showing the results of performing real-time reverse transcription PCR using a dry mixture at one-day intervals for a storage period of a total of 5 days after storage at 50°C in order to test the storage stability of the dry mixture:

1 day to 5 days: the number of days during which the dry mixture was stored at 50°C.

FIG. 9a shows the results of performing real-time reverse transcription PCR using a primer having 6 mismatch nucleotides at the 5' end, and FIG. 9b shows the results of performing real-time reverse transcription PCR using a primer having 6 mismatch nucleotides at the middle. Also, FIGS. 9a and 9b graphically show the results of performing real-time reverse transcription to confirm the inhibition of non-specific binding of a primer to a template in a hot-start

reverse transcription PCR reaction mixture.

FIGS. 10a and 10b are graphs showing the results of performing real-time reverse transcription PCR in order to analyze the single nucleotide polymorphism of RNA by hot-start reverse transcription PCR:

"A" indicates the result obtained using a primer having an exactly matched nucleotide sequence, and "B" indicates the result obtained using a primer having a single mismatch nucleotide.

FIG. 11 graphically showing the results of performing real-time reverse transcription in order to examine whether a hot-start reverse transcription reaction inhibits non-specific reactions resulting from RNA-RNA self-priming:

"A" in FIG. 11 shows the results of performing a real-time PCR using RNA alone without a reverse transcription reaction; and "B" shows the results of performing a reverse transcription reaction using a primer, followed by real-time PCR. "B-A" indicates the result obtained by performing the reverse transcription reaction using a hot-start reverse transcription reaction solution, and "B-B" shows the result obtained by performing the reverse transcription reaction using a reaction solution having no hot-start reverse transcription function. "C" shows the results of performing a reverse transcription reaction using RNA alone in a reaction solution having no hot-start reverse transcription function, followed by real-time PCR. "D" shows the results of performing a reverse transcription reaction using RNA alone in a hot-start reverse transcription reaction solution, followed by real-time PCR.

FIG. 12 is a graphic diagram showing the results of performing real-time reverse transcription PCR in order to examine whether RNA-RNA self-priming in a reverse transcription reaction is inhibited by RNA termination. "A" and "B" indicates the result obtained using non-terminated RNA, and "C" and "D" indicate the results obtained using terminated RNA. For "A" and "C", a plain reverse transcription reaction was performed, and for "B" and "D", a hot-start reverse transcription reaction was performed.

FIG. 13a shows the results of detecting the cancer marker keratin-8 using RNA extracted from Hela cells. For quantitative analysis, the extracted RNA was serially 10-fold diluted and was subjected to real-time reverse transcription PCR in order to plot a standard curve. FIGS. 13b, 13c, 13d and 13e are graphs showing the results of performing real-time reverse transcription PCR in order to examine whether RNA termination and hot-start reverse transcription PCR increase the detection limit of the cancer marker.

BEST MODE FOR CARRYING OUT THE INVENTION

[0017] In one aspect, the present invention is directed to a composition for hot-start reverse transcription reaction, which comprises an Mg^{2+} ion, four kinds of dNTPs, reverse transcription polymerase, pyrophosphate (PPi), and pyrophosphatase (PPase).

[0018] The Reverse transcription polymerase that is used in the present invention may be any known reverse transcription polymerase. Specifically, commercially available AMV or MMLV may generally be used.

[0019] As pyrophosphatase (PPase), commercially available conventional PPase may be used in the present invention without limitation. Specifically, PPase that is used in the present invention may preferably be Tte-inorganic pyrophosphatase (SibEnzyme Ltd.) derived from an *E. coli* cloned with an inorganic pyrophosphatase gene derived from Thermus thermophilus B35, or Pto-inorganic pyrophosphatase (Bioneer corporation, Korea) derived from an *E. coli* cloned with an inorganic pyrophosphatase gene derived from Picrophilus torridus. 1 unit of Pto-inorganic pyrophosphatase is defined as the amount of the enzyme required to produce 40 nmole of phosphate from pyrophosphate for 1 minute. The reaction is performed using Tris-HCl (pH 7.5), 5 mM $MgCl_2$, and 2.0 mM PPi with a total reaction volume of 0.5 ml at 70°C for 10 minutes.

[0020] PPi that is contained in the composition may be added at a concentration of 0.1-5.0 mM, preferably 0.2-3.0 mM, based on the final reaction solution. If the concentration of PPi is higher than 5.0 mM, the concentration of PPase to be used needs to increase proportionately, and in this case, the amount of the reverse transcription reaction product can decrease. If the concentration of PPi is lower than 0.1 mM, the ability to capture the Mg^{2+} ion in the reverse transcription reaction composition will be reduced, and thus the effect of inhibiting the production of non-specific products caused by non-specific priming (mispriming) cannot be obtained. When the concentration of PPi is 0.1-5 mM, 0.005-0.25 U of PPase is preferably contained in the reverse transcription reaction mixture. PPase is used 0.005-0.25 U per 0.1 mM PPi. If PPase is used at a concentration higher than 0.25 U per 0.1 mM PPi, the result can be confirmed at a higher concentration when a high-copy-number template is used, compared to when a low-copy-number template is used, and the reverse transcription reactivity can be reduced, resulting in a significant decrease in the amount of the reaction product. If the concentration of PPase is lower than 0.005 U per 0.1 mM PPi, the reverse transcription reaction can be inhibited.

[0021] The reaction buffer solution that is used in the present invention is preferably a buffer (pH 9.0) containing 10 mM Tris HCl and 40 mM KCl, and the four kinds of dNTPs indicate dATP, dTTP, dGTP and dCTP. If necessary, the composition may further comprise substances required for reverse transcription reaction, including primers, template nucleic acid and the like. The primers may preferably be random primers, or primers specific for the template nucleic acid, and the template nucleic acid is preferably an RNA in need of a reverse transcription reaction. Additionally, the composition may comprise a fluorescent dye that may be selected from the group consisting of SyBr Green, EtBr and HRdye.

[0022] The composition for reverse transcription reaction according to the present invention may further comprise reverse transcription primers. The reverse transcription primers that are used in the present invention may be any primers that can anneal the template RNA to perform the reverse transcription of the RNA. Specifically, the reverse transcription primers may be poly A primers, or primers specific to the template RNA.

[0023] Meanwhile, the composition for reverse transcription reaction according to the present invention may comprise a dye and/or a polyol, which is not reactive with nucleic acid, for the purposes for the convenience of experiments, the prevention of contamination by PCR products, the stabilization of DNA polymerase and dNTPs and the improvement of reactivity.

[0024] The "non-reactive dye" should be selected from among substances that do not influence reverse transcription reactions, and it is used to analyze or identify PCR reactions using reverse reaction products. Examples of substances that satisfy such conditions include water soluble dye such as rhodamine, tamra, lax, bromophenol blue, xylene cyanole, bromocresol red, and cresol red. Among them, xylene cyanole is preferably used. The non-reactive dye may be contained in an amount of 0.0001-0.01 wt%, preferably 0.001-0.005 wt%, and more preferably 0.001-0.003 wt%, based on the total weight of the composition. If the content of the non-reactive dye in the composition is less than 0.0001 wt%, it will be difficult to visually observe the migration of a sample during agarose gel electrophoresis for analysis after the reverse transcription reaction, because the concentration of the dye is low. If the content of the non-reactive dye in the composition is higher than 0.01 wt%, a high concentration of the water-soluble dye can act as a reaction inhibitor during the reverse transcription reaction. In addition, this dye can interfere with the migration of the sample during agarose gel electrophoresis.

[0025] Also, the polyol can be used as an additional stabilizer for further stabilizing the composition of the present invention, and may be one or more compounds selected from the group consisting of glucose, glycerol, mannitol, galacitol, glucitol, and sorbitol. The content of the polyol in the composition may be 10-500 mM, and preferably 50-300 mM. If the content of the polyol is higher than 500 mM, it will be difficult to dissolve with an aqueous solution, due to the solubility of the water-soluble polymer itself, will be difficult to mix sufficiently, due to its high viscosity, will increase the volume of the composition to a volume greater than required, and will not be easily dissolved in a sterile distilled water and gene solution before the reverse transcription reaction. In addition, a high-concentration of the water-soluble polymer can act as a reaction inhibitor during the reverse transcription reaction. On the other hand, if the content of the polyol in the composition is less than 10 mM, the target enzyme and surface water molecules cannot be sufficiently coated so that they cannot be protected, and thus the effect of efficiently stabilizing the enzyme cannot be obtained. In addition, the solution can spread throughout the bottom of the tube due to its too low viscosity and cannot be dried in an ideal form, and the enzyme cannot be sufficiently protected. In the present invention, in addition to the polyol, gelatin, bovine serum albumin, Thesit or PEG-8000 may be used as a stabilizer.

[0026] The composition of the present invention is preferably freeze-dried or dried in order to increase the stability, the convenience of storage and the long-term storage stability thereof. The drying may be performed by a known drying method, such as room temperature drying, drying at elevated temperature, for example, 40 to 60°C, freeze drying, or reduced pressure drying. In addition, any drying method may be used, as long as the component of the composition is not lost. The drying method as described above may be applied depending on the kind and amount of enzyme used. In the present invention, the freezing drying method or the reduced pressure method employing vacuum centrifugation may preferably be used.

[0027] The composition for hot-start reverse transcription PCR may be prepared in a stable form by introducing the reaction mixture into a single reaction tube, and then immediately, freezing or drying the reaction mixture.

[0028] If necessary, the reaction mixture may further comprise a primer, a probe, template nucleic acid, a fluorescent dye, a non-reactive dye and/or a polyol.

[0029] As the DNA polymerase, any known DNA polymerase may be used in the present invention without particular limitation. Among these polymerases, a polymerase having 5'->3' exonuclease activity, a polymerase having 3'->5' exonuclease activity, and a polymerase that does not have 5'->3' exonuclease activity and 3'->5' exonuclease activity may be used alone or in combination. Examples of the polymerase having 5'->3' exonuclease activity include Taq DNA polymerase, examples of the polymerase having 3'->5' exonuclease activity include Pfu DNA polymerase or TLA DNA polymerase (Bioneer), and examples of the polymerase having no 5'->3' exonuclease activity and 3'->5' exonuclease activity include Top DNA polymerase (Bioneer). The DNA polymerase may be contained in the composition for reverse transcription PCR at a concentration of 0.1-10 U (unit), preferably 0.5-2 U, and most preferably 1 U. The Taq, Pfu, Top

and TLA DNA polymerases have the characteristics shown in Table 1 below.

Table 1

	Taq. DNA polymerase	Pfu DNA polymerase	Top DNA polymerase	TLA DNA polymerase
5'->3' exonuclease activity	Yes	No	No	No
3'->5' exonuclease activity	No	Yes	No	Yes
End transferase activity	Yes	No	Yes	No
Error rate ($x10^{-6}$)	4.91	1. 90	unconfirmed	unconfirmed
Size of segments	≤ 10 kbp	≤ 5 kbp	≤ 10 kbp	≤ 15 kbp
Optimum activity temperature (°C)	72	72	72	72
Half life(min, at 95°C) $MgCl_2$(mM)	80 1.5	unconfirmed	unconfirmed 1.5	unconfirmed 1.0
$MgSO_4$(mM)	-	2.0	-	-
KCl (mM)	40	10	30	70
optimum pH(at 25°C)	9.0	8.8	9.0	9.0

[0030] In addition, the composition for reverse transcription PCR may be used in, in addition to reverse transcription PCR, any nucleic acid amplification reaction such as multiplex reverse transcription PCRs, real-time reverse transcription PCR, real-time quantitative PCR or multiple real-time reverse transcription PCRs.

[0031] In the present invention, the composition for reverse transcription PCR may further comprise a substance that shows a hot-start PCR effect by binding to DNA polymerase or regulating the action of DNA polymerase. This substance may be selected from the group consisting of an antibody, an aptamer and an affibody, which bind to DNA polymerase.

[0032] In the present invention, the DNA polymerase may be in a modified form and may have a hot-start PCR effect, and examples of this enzyme include, but are not limited to, Tth polymerase.

[0033] PPase is preferably a heat-stable enzyme that is thermally stable even at a temperature of 70°C or above. It is thermally stable during the reverse transcription and PCR processes. Particularly, PPase shows enzymatic activity at a temperature similar to the temperature at which reverse transcriptase and DNA polymerase react. For this reason, reverse transcription reaction and PCR are prevented from occurring during the mixing process and PPase is reacted to decompose PPi at a temperature equal to or higher than the temperature at which primers specifically hybridize, whereby reverse transcriptase can be activated while reverse transcription reaction can be initiated at the hybridization temperature of the primers, to eliminate non-specific cDNA synthesis. In addition, when the DNA polymerase hybridizes, non-specific nucleic acid amplification can be prevented. As a result, selectivity can be greatly increased compared to that of conventional reverse transcription/PCR, and a very small amount of the target nucleic acid can be successfully detected. Thus, the inventive composition for hot-start reverse transcription reaction or reverse transcription PCR comprising PPi and PPase is a stable composition that can solve problems of non-specific reverse transcription reaction occurring in conventional reverse transcription reaction, and the resulting non-specific PCR amplification, and that can specifically amplify a desired target product without troublesomeness to provide an accurate amplification result.

[0034] Specifically, the composition for hot-start reverse transcription reaction according to the present invention has the following advantages over various reverse transcription reaction compositions that have been reported in the prior art and have been commercially used:

1) Even when a target RNA is contained in a very small amount in the total RNA of a reaction solution, the composition for hot-start reverse transcription PCR according to the present invention can selectively perform the reverse transcription of the target RNA to perform PCR amplification in a manner specific to the target RNA. Thus, the composition of the present invention it makes it possible to detect a very small amount of RNA virus or analyze a cancer-related RNA gene.
2) The composition of the present invention can prevent the production of amplification products caused by non-specific priming, and thus can simultaneously detect various targets in multiplex reverse transcription PCRs in which

reverse transcription PCR reactions for various targets are simultaneously performed.

3) Because all the components of the reaction mixture for reverse transcription PCR, which are used in the present invention, are prepared as a single mixture, a separate mixing process is not required during reverse transcription PCR, and thus the occurrence of error caused by mixing during the reaction can be prevented. In addition, the occurrence of non-specific PCR products can be prevented, the experiment can be conveniently performed, contamination by reverse transcription PCR reaction products can be prevented, and the stability and reactivity of reverse transcription polymerase, DNA polymerase and dNTPs can be increased.

4) When nucleic acid is used in a mixture with a stabilizer, stability is increased, it is easy to use and convenient to store.

[0035] In a preferred embodiment of the present invention, in order to solve the problems of non-specific reverse transcription reactions occurring in the prior art and perform hot-start reverse transcription reaction in a target-specific manner, only a desired RNA into cDNA is synthesized and PPi and PPase were used in the reverse transcription reaction to perform a PCR reaction in a manner specific to the synthesized cDNA to thereby amplify a desired RNA with high sensitivity. Specifically, 0.5 mM to 2.0 mM (preferably 2.0 mM) of PPi and 0.005 U to 0.25 U, preferably 0.1 U of PPase were added to a reverse transcription reaction mixture containing reverse transcription polymerase, and then a reverse transcription reaction was performed using the reaction mixture at 42°C for 60 minutes. As a result, it was shown that, when no PPase was added, reverse transcription reactions were inhibited by PPi (see FIG. 1), but PPi and PPase were added, reverse transcription and PCR reactions were activated to increase the amount of the reaction product (see FIGS. 2 and 3). In addition, it was shown that, when the gene of hepatitis C virus was amplified using PPi and PPase, a template RNA having a copy number of 10^{10} to 10 could be detected (see FIG. 4), and when the total RNA extracted from human cells, in addition to the template RNA of hepatitis C virus, was added, non-specific reactions caused by the human RNA were inhibited, and RNAs other than the target RNA did not result in a decrease in reaction efficiency and changes in sensitivity and detection ability. However, in the absence of PPi and PPase, non-specific reactions were caused by the added human RNA (see FIGS. 5a and 5b). Moreover, it was shown that, in the hot-start reverse transcription PCR method, non-specific reactions were significantly reduced compared to those in conventional hot-start PCR methods employing Taq antibody, and thus a target RNA with even a copy number of 10 was selectively detected with high sensitivity (see FIGS. 6a and 6b). In addition, the composition for hot-start reverse transcription PCR according to the present invention was dried to increase the stability and storage stability thereof, and it was found that the dried composition could stably show reactivity and amplification results similar to those of the composition that was not dried (see FIGS. 7 and 8).

[0036] Accordingly, the composition for hot-start reverse transcription reaction and the composition for reverse transcription PCR can be used to perform high-sensitivity reverse transcription reaction and PCR, and thus can be advantageously used in RNA amplification technologies for examination of various viruses and examination of cancer gene expression.

[0037] In the present invention, in order to prevent the template nucleic acid itself from being non-specifically self-primed and amplified in nucleic acid amplification reactions such as a PCR reaction or real-time quantitative PCR reaction for detecting the target nucleic acid, a method may additionally be used, in which a substance for terminating polymerization reaction and a nucleic acid polymerase are added to the template nucleic acid to perform a termination reaction so that the template nucleic acid cannot act as a primer, and thus only the target nucleic acid can be amplified and detected with high sensitivity.

[0038] Thus, the composition of the present invention can be characterized in that it further comprise a template nucleic acid that has a nucleic acid polymerization terminator bound to the 3' end to prevent non-specific nucleic acid polymerization. The nucleic acid polymerization terminator may be a nucleic acid-like compound that is activated in the form of triphosphate capable of acting on nucleic acid polymerase and has groups other than a hydroxyl group at the 3' end.

[0039] In the present invention, the template nucleic acid that has the nucleic acid polymerization terminator bound thereto to prevent non-specific nucleic acid polymerization is prepared by the following nucleic acid preparation method comprising the steps of: 1) terminating the 3' end of the template nucleic acid with a reaction mixture containing nucleic acid polymerase, enzymatic reaction buffer and a nucleic acid polymerization terminator; and 2) inactivating or removing the nucleic acid polymerization terminator from the reaction mixture.

[0040] More specifically, the nucleic acid preparation method may comprise the steps of: 1) separating and purifying nucleic acid from a biological sample containing the nucleic acid; 2) terminating the 3' end of the separated and purified template nucleic acid of step 1) with a reaction mixture containing nucleic acid polymerase, enzymatic reaction buffer and a nucleic acid polymerization terminator; and 3) inactivating or removing the nucleic acid polymerization terminator from the reaction mixture containing the nucleic acid terminated at the 3' end.

[0041] The nucleic acid preparation method described in the present invention may be performed using a known conventional nucleic acid extraction device, for example, a device described in Korean Patent No. 10-0148239, US 5702590, US 5647994, EP 0691541, US 5336760, US 5897783, US6187270, or Korean Patent Application No. 10-2008-0032904. In addition, ExiPrep™ 16-fully Automated DNA/RNA/Proteins Purification System (Bioneer, Korea)

may preferably be used as an automatic nucleic acid extraction device in the present invention.

[0042] In the present invention, the sample may be a sample that contains nucleic acid (polynucleotide) and that is derived from any plant, animal, bacterium or virus.

[0043] In the present invention, the nucleic acid is RNA or DNA, and the nucleic acid polymerase comprises any nucleic acid polymerase that enables a terminator to be attached to the 3' end of DNA or RNA so as to terminate polymerization.

[0044] More specifically, the nucleic acid polymerase that is used in the present invention is preferably a polymerase that has no 3' → 5' exonuclease activity so as not to hydrolyze the terminator. More preferably, it is a polymer that loses its enzymatic activity at a temperature of 90°C or higher. The nucleic acid polymerase may comprise at least one selected from among RNA-dependent RNA-polymerase, RNA-dependent DNA-polymerase, DNA-polymerase, and RNA-polymerase.

[0045] As used herein, the term "nucleic acid polymerization terminator" refers to a substance that terminates the polymerization of all the fragments of the template nucleic acid so that the nucleic acid no longer elongates. It also refers to a substance that is bound to the end of the template nucleic acid fragment so that the chain elongation of the nucleic acid can no longer occur.

[0046] As used herein, the term "termination" means that the "nucleic acid polymerization terminator" is covalently bound to the 3' end of nucleic acid to terminate the polymerization of the nucleic acid, and the substance that is used for termination is defined as a terminator.

[0047] More specifically, the term "termination" as mentioned herein means that a substance having no hydroxyl group at 3' end or having a chemical group that terminates a polymerization reaction caused by polymerase is covalently bound to the 3' end of nucleic acid to terminate the polymerization of the nucleic acid, and the substance that is used for termination is defined as a terminator.

[0048] In the present invention, the nucleic acid terminator is a nucleic acid-like compound activated in the form of triphosphate capable of acting on nucleic acid polymerase and may be selected from among various nucleotide triphosphates that lack the 3' hydroxyl group to which nucleic acid is linked or which are substituted with other groups. It may be a nucleic acid terminator having good reactivity with nucleic acid polymerase.

[0049] The nucleic acid-like compound that can be used in the present invention may comprise at least one selected from among 2'3'-dideoxynucleoside 5'-triphosphate, 3'-deoxyadenosine 5'-triphosphate, 3'-azido-3'-deoxythymidine 5'-triphosphate, 1-β-d-Arabinofuranosylnucleoside 5'-Triphosphate, acyclo-guanosine triphosphate, 3'-amino-2'-deoxynucleoside 5'-triphosphate, and 3'-fluoro-3'-deoxynucleoside 5'-triphosphate.

[0050] More specifically, DNA-polymerase derived from *E. coli* or heat resistance bacteria may be 2'3'-dideoxynucleotide triphosphate (ddNTP) that has been frequently used in the dideoxy sequencing method developed by Sanger.

[0051] In the present invention, the dideoxynuclreotide triphosphate (ddNTP) may comprise at least one selected from among dideoxyguanosine triphosphate (ddGTP), dideoxyadenosine triphosphate (ddATP), dideoxythymidine triphosphate (ddTTP), and dideoxycytidine triphosphate (ddCTP).

[0052] The deoxynuclreotide triphosphate (dNTP) may comprise at least one selected from among guanosine, adenosine, thymidine, uridine and cytidine.

[0053] For example, various nucleic acid polymerization terminators may be used to perform a termination reaction using DNA-polymerase. When DNA-polymerase derived from mammalian is used, it is possible to use 5'-triphosphate 1-β-d-arabinofuranosylcytosine or 5'-triphosphate 9-β-d-arabinofuranosyladenine, which has high reactivity with this enzyme (Inhibition of Mammalian DNA Polymerase by the 5'-Triphosphate of 1-β-d-Arabinofuranosylcytosine and the 5'-Triphosphate of 9-β-d-Arabinofuranosyladenine, J. J. Furth, and Seymour S. Cohen, Cancer Res October 1968 28; 2061).

[0054] In addition, acyclonucleoside triphosphate that has frequently been used as an antiviral agent for inhibiting viral DNA-polymerases may also be used as the nucleic acid polymerization terminator (Inhibition of herpes simplex virus-induced DNA polymerase activity and viral DNA replication by 9-(2-hydroxyethoxymethyl)guanine and its triphosphate. P A Furman, M H St Clair, J A Fyfe, J L Rideout, P M Keller and G B Elion, J. Virol. October 1979 vol. 32 no. 1 72-77).

[0055] In addition, poly A polymerase that is RNA polymerase is an enzyme that is used to attach adenosine to the 3' end of RNA and is suitable for the nucleic acid polymerization termination as described in the present invention. When this enzyme is used, 3'-deoxyadenosine triphosphate (3'-dATP; cordycepin) known as an inhibitor of poly A polymerase may be used.

[0056] In the present invention, step 2) is a process of inactivating or removing unreacted nucleic acid polymerization terminator after step 1), in which inactivation of the nucleic acid polymerization terminator may be performed using any enzyme capable of hydrolyzing the triphosphate bond of various nucleic acid polymerization terminators that are used in polymerization.

[0057] More specifically, the nucleic acid terminator is preferably easily inactivated by heat, because it loses its enzymatic function after it inactivates the nucleic acid polymerization terminators by hydrolysis. This is because it does not degrade nucleotide triphosphate in a subsequent reverse transcription reaction or PCR reaction.

[0058] For example, the phosphatase may be BAP (bacterial alkaline phosphatase) or CIP (calf intestinal phosphatase), which has the property of degrading triphosphate. Preferably, CIP that is easily inactivated by heat is used.

[0059] In addition, an alkaline phosphatase (Autotaxin) (Clair T, Lee HY, Liotta LA, Stracke ML (1997). "Autotaxin is an exoenzyme possessing 5'-nucleotide phosphodiesterase/ATP pyrophosphatase and ATPase activities". J. Biol. Chem. 272 (2): 996-1001. doi:10.1074/jbc.272.2.996. PMID 8995394.) may be used, which hydrolyzes pyrophosphate and ATP and is derived from various organisms, including *E. coli,* bovine small intestine, and shrimps. Also, *Staphylococcus aureus* adenosine synthase (AdsA) that non-specifically degrades nucleotide triphosphate may be used.

[0060] In the nucleic acid preparation method according to the present invention, subsequently to the nucleic acid separation/purification process, nucleic acid polymerase and a nucleic acid polymerization terminator may be added to the extracted nucleic acid to terminate the polymerization of the nucleic acid to thereby prevent the non-specific priming of the template nucleic acid. After the reaction, a step of removing unreacted nucleic acid polymerization terminators is required, because the unreacted nucleic acid polymerization terminators act as an inhibitor of a subsequent polymerase chain reaction or other PCR reactions.

[0061] Removal of the nucleic acid polymerization terminator may be performed using gel filtration, or a chaotropic agent and a silica bead, but is not limited thereto.

[0062] The nucleic acid preparation method according to the present invention may be performed using a known nucleic acid analyzer comprising a known automatic nucleic acid extraction system and a gene amplification system. Preferably, Exiprep 16 DX (Bioneer, Korea) as described above may be used, but is not limited thereto.

[0063] More specifically, subsequently to the nucleic acid separation/purification process, nucleic acid polymerase and a nucleic acid terminator may be added to the extracted nucleic acid to terminate the polymerization reaction of the nucleic acid and inactivate the priming function of all the nucleic acids. The terminator remaining after the termination acts as an inhibitor of a subsequent reverse transcription reaction or PCR reaction, and for this reason, two methods can be used to remove the remaining terminator: a method of performing RNA purification again using the Exiprep 16 DX system (Bioneer); and a method of degrading the nucleic acid polymerization terminator so as to no longer react.

[0064] The method of performing RNA purification using the Exiprep 16 DX system is a method in which RNA is attached to magnetic silica particles in the presence of a chaotropic agent and washed and eluted. This method has problems in that it is time-consuming because several steps are performed and in that the purified RNA is lost. On the other hand, the method of inactivating the activated nucleic acid polymerization terminator (nucleotide triphosphate) by degrading it into di- or mono-triphosphates has many advantages in that it is fast and simple and does not reduce the yield.

[0065] More specifically, in the method of inactivating the nucleic acid polymerization terminator using enzyme, the wells of Exiprep 16 DX, which contain magnetic particles and are used in the elution step, are temperature-controllable wells. Thus, after elution with elution buffer, the nucleic acid polymerization terminator can be easily can be removed by adding triphosphate hydrolase, such as alkaline phosphatase, autotoxin or *Staphylococcus aureus* adenosine synthase (AdsA) to the wells and reacting the enzyme at 37°C.

[0066] In another aspect, the present invention also relates to a method for preparing a composition for hot-start reverse transcription reaction or hot-start reverse transcription PCR.

[0067] Specifically, the preparation method comprises introducing a reaction mixture, which comprises a reaction buffer solution, $MgCl_2$, four kinds of dNTPs, reverse transcription polymerase, PPi and PPase, into a single reaction tube. Particularly, in the preparation of the composition for hot-start reverse transcription PCR, the reaction mixture further comprises DNA polymerase.

[0068] The DNA polymerase may be one or more selected from the group consisting of a polymerase having 5'->3' exonuclease activity, a polymerase having 3'->5' exonuclease activity, and a polymerase that does not have 5'->3' exonuclease activity and 3'->5' exonuclease activity.

[0069] Preferably, the above preparation method further comprises a step of freezing or drying the reaction mixture to form a dry composition having increased stability and long-term storage stability, but is not limited thereto.

[0070] The pyrophosphate (PPi) is preferably contained at a concentration of 0.1-5 mM, and preferably 0.5-2.0 mM, and the pyrophosphatase is preferably contained in an amount of 0.005-0.25 U per 0.1 mM PPi, but is not limited thereto.

[0071] Additionally, the reaction mixture may comprise at least one selected from the group consisting of one or more primers or probes, a fluorescent dye that binds to DNA, template nucleic acid, a dye non-reactive with nucleic acid, a polyol, gelatin, bovine serum albumin, Thesit, and PEG-8000. Herein, the fluorescent dye may be selected from the group consisting of SyBr Green, EtBr and HRdye, and the dye may be one or more selected from the group consisting of rhodamine, tamra, lax, bromophenol blue, xylene cyanole, bromocresol red, and cresol red.

[0072] In still another aspect, the present invention is directed to a kit for hot-start reverse transcription reaction, which comprises the above-described composition for hot-start reverse transcription reaction.

[0073] The present invention is also directed to a kit for hot-start reverse transcription PCR, which comprises the above-described composition for hot-start reverse transcription PCR.

[0074] The kit may be prepared according to a conventional method for preparing a kit for reverse transcription reaction or a kit for reverse transcription PCR.

[0075] The present invention is also directed to a method of reverse transcription of template RNA, the method comprising the steps of: mixing the composition for hot-start reverse transcription reaction with a sample containing the template RNA to form a reaction mixture; and performing a reaction so that the reaction mixture can be reverse-transcribed.

[0076] The present invention also provides a method of amplifying nucleic acid, the method comprising the steps of: mixing the composition for hot-start reverse transcription PCR with a sample containing template RNA to obtain a reaction mixture; performing a reaction so as to amplify the reaction mixture to thereby obtain an amplification product; and analyzing the amplification product.

[0077] Herein, the reverse transcription PCR reaction may be any one or more of multiplex reverse transcription PCRs, real-time reverse transcription PCR, and real-time quantitative reverse transcription PCR.

[0078] In a preferred embodiment of the present invention, the template nucleic acid is preferably an RNA that may be amplified by reverse transcription and PCR.

[0079] The inventive composition for hot-start reverse transcription reaction and the inventive composition for reverse transcription PCR comprise, in addition to a reverse transcription reaction mixture comprising reaction buffer solution, $MgCl_2$, four kinds of dNTPs and reverse transcription polymerase, PPi and PPase, which can prevent a reverse transcription reaction from occurring in a mixing process at room temperature. In addition, PPi can be degraded to activate the reverse transcription polymerase by reacting PPase at a temperature equal to or higher than the temperature at which primers specifically hybridize, and thus a reverse transcription reaction can be initiated at the hybridization temperature so that non-specific cDNA synthesis can be eliminated. Thus, selectivity can be greatly increased compared to that of conventional reverse transcription reactions, and even a very small amount of the target RNA can be successfully detected. In addition, when the composition is frozen, or dried in a mixture with a stabilizer, it can be used in a stable and convenient manner compared to conventional compositions, and thus is useful as a more stable composition for hot-start reverse transcription reaction.

[0080] In an embodiment of the present invention, a hot-start reverse transcription was performed in the presence of PPi and PPase using a reverse primer exactly matching the template RNA and a primer having a nucleotide sequence mismatching the template RNA. As a result, the amplification product was detected in the reverse primer exactly matching the template RNA, but was not detected in the reverse primer having the nucleotide sequence mismatching the template RNA. On the contrary, it was found that, when a hot-start reverse transcription reaction was performed in the absence of PPi and PPase, the amplification product was detected even in the reverse primer having the mismatch nucleotide sequence.

[0081] In another embodiment of the present invention, the single-nucleotide polymorphism of the target RNA was analyzed using a hot-start reverse transcription PCR reaction. As a result, it was found that, when the hot-start reverse transcription reaction was performed in the presence of PPi and PPase, the results obtained using the primer exactly matching the template RNA did differ in PCR efficiency from the results obtained using the reverse primer having a single mismatch nucleotide. However, the hot-start reverse transcription reaction having no PPi and PPase was detected in the same cycle without difference between the matching reverse primer and the mismatch reverse primer. This suggests that the hot-start reverse transcription reaction according to the present invention makes it possible to analyze single-nucleotide polymorphism using a real-time PCR method, but in a reaction solution having no hot-start reverse transcription function, it is difficult to analyze single-nucleotide polymorphism.

[0082] In another embodiment of the present invention, the effect of the hot-start reverse transcription PCR reaction of the present invention on the inhibition of non-specific reactions resulting from the self-priming of single-stranded nucleic acids contained in extracted nucleic acids was examined. As a result, it was found that the hot-start reverse transcription reaction inhibited non-specific reactions resulting from self-priming, compared to a reaction solution having no hot-start function (that is, having no PPi and PPase).

[0083] In another embodiment of the present invention, the effect of RNA termination on the inhibition of non-specific reverse transcription PCR reactions resulting from RNA self-priming was examined. It was shown that, when an extracted RNA terminated with poly(A) polymerase and 3'-deoxyadenosine 5'-triphosphate (3'-dATP) at the 3' end was used as a template, cDNA was synthesized from non-terminated RNA even when any primer was not added. In other words, the cDNA was synthesized by self-priming and amplified in PCR. Also, the PCR amplification resulting from the non-specific cDNA synthesis could not be perfectly prevented even by the hot-start reverse transcription PCR reaction, and the reverse transcription PCR reaction by RNA self-priming could be partially inhibited, and thus the Ct value was reduced by about 5, indicating that the non-specific reaction could be inhibited by about 1/30.

[0084] However, it was shown that, in a sample comprising the RNA terminated at the 3' end, cDNA was not amplified up to 45 cycles in all the reverse transcription PCR reactions, indicating that no cDNA was synthesized. Such results suggest that many non-specific reverse transcription PCR reactions occur during mixed reverse transcription PCR reaction mixture, and for this reason, many non-specific cDNAs are made. Also, from the above results, it can be seen that undesired reactions cannot be inhibited by the hot-start reverse transcription PCR alone. As a result, it can be seen that such non-specific reverse transcription PCR reactions resulting from RNA self-priming can be perfectly inhibited by

terminating the RNA using the termination reaction of the present invention.

[0085] In another embodiment of the present invention, it was found that, when the hot-start PCR of the present invention is used, the detection limit of a cancer marker is increased.

[0086] The nucleotide sequence information of Homo sapiens Keratin 8 (KRT8) as a cancer marker was obtained from the National Center for Biotechnology Information (NCBI, USA), and RNA was extracted from human Hela C cells and subjected to RNA termination. As a result, in the case of samples subjected to RNA termination, the cancer marker was detected in a hot-start reverse transcription reaction sample containing 10 or more Hela cells added to 1 ml of human serum. In the case of reactions having no hot-start reverse transcription reaction function, the caner marker was detected in the sample containing 100 or more Hela cells. In the case of samples comprising non-terminated RNA, the cancer marker was detected in hot-start reverse transcription reaction samples containing 100 or more Hela cells. In the case of reaction solutions having no hot-start reverse transcription function, the caner marker was detected in the samples containing 1,000 or more Hela cells.

[0087] Thus, it can be seen that the detection limit was increased even by the hot-start reverse transcription reaction alone, but a better effect could be obtained when the hot-start reverse transcription reaction was performed together with RNA termination.

[0088] Although the present invention has been described in detail with reference to the specific features, it will be apparent to those skilled in the art that this description is only for a preferred embodiment and does not limit the scope of the present invention. Thus, the substantial scope of the present invention will be defined by the appended claims and equivalents thereof.

EXAMPLES

[0089] Hereinafter, the present invention will be described in further detail with reference to examples. It is to be understood, however, that these examples are for illustrative purposes only and are not intended to limit the scope of the present invention.

Example 1: Examination of the effect of PPi on inhibition of reverse transcription reactions

[0090] In order to examine the conditions in which a reverse transcription reaction is inhibited by PPi, a reverse transcription reaction was performed using 1.5 mM magnesium ion and varying concentrations of PPi, and the reaction product was subjected to PCR. Specifically, 0.5-2 mM of PPi was added to Accupower RT Premix (Bioneer, Korea), and a reverse transcription reaction and a PCR reaction were performed. The reverse transcription reaction was performed using 100 ng, 10 ng, 1 ng or 100 pg of RNA extracted from human Hela cells was added, in the presence of PPi added at a concentration of 0, 0.5, 1, 1.5, 2 or 2.5 mM. The reverse transcription reaction was performed once at 42°C (the optimum temperature of Accupower RT Premix (Bioneer, Korea)) for 60 minutes, and was performed once at 95°C for 5 minutes to inactivate reverse transcriptase. 5 $\mu\ell$ of 20 $\mu\ell$ of the reverse transcription reaction product was added to Accupower PCR Premix (Bioneer, Korea) and subjected to a PCR reaction using the GAPDH forward primer 5'-GGAAGGTGAAGGTCGGAGTC-3' (SEQ ID NO: 1) and the GAPDH reverse primer 5'-GCCAAATTCGTTGTCATACC-3' (SEQ ID NO: 2), which target the nucleotide sequences of human GAPDH primers. The PCR reaction was performed under the following conditions: pre-denaturation at 95°C for 5 min, and then 30 cycles, with 95°C for 20 sec, 55°C for 40 sec, and 72°C for 60 sec as one cycle, are followed by final extension at 72°C for 5 min. The PCR reaction product was electrophoresed on agarose gel together with a DNA molecular weight marker, and then stained with ethidium bromide, and the DNA band amplified by the PCR reaction was photographed with a Polaroid camera. As a positive control, RT PreMix (Bioneer, Korea) containing no PPi was used, and the product was subjected to a PCR reaction.

[0091] As a result, it could be seen that, in the experimental group containing PPi, the reverse transcription reaction was inhibited from a PPi concentration of 0.5 mM and was more clearly inhibited when 2 mM PPi was added (FIG. 1).

Example 2: Examination of reverse transcription by PPase that hydrolyzes PPi into two phosphates

[0092] The results of Example 1 indicated that reverse transcription reactions were inhibited by addition of PPi. For the reverse transcription reaction as described above, PPase that degrades PPi into two phosphates was added in order to examine the activation of the reverse transcription reaction. Specifically, under the condition in which PPi was added in the same manner as described in Example 1, 0.1 U of PPase was added, and a PPi enzyme reaction and a reverse transcription reaction were simultaneously performed at 42°C for 60 minutes. Then, the reaction product was allowed to stand at 95°C for 5 minutes in order to inactivate the reverse transcriptase, and was then subjected to PCR.

[0093] As a result, it was found that, when PPase was added, PCR was activated to maximize the amount of the PCR product (FIG. 2).

Example 3: Examination of real-time reverse transcription PCR in the presence of PPi and PPase

[0094] In order to examine a real-time reverse transcription PCR reaction by a composition containing PPi and PPase, 2 mM PPi and 0.1 U PPase were added 2X PCR PreMix solution (containing 10 mM Tris HCl pH 9.0, 50 mM KCl, 1.5 mM $MgCl_2$, four kinds of dNTPs (each 250 uM), 1U taq DNA polymerase, 200 U reverse transcriptase, 1 mM DTT, 0.01% Tween 20, and stabilizer), and a reaction was performed using 25 $\mu\ell$ of the PCR PreMix solution. The forward primer 5'-CGTGGAAGGACTCATGACCACA-3' (SEQ ID NO: 3), the reverse primer GCCTTGGCAGCGCCAGTAGA-3' (SEQ ID NO: 4) and the GAPDH probe 5'-CTGTGGATGGCCCCTCCGGGAAA-3' (SEQ ID NO: 5), which target the human GAPDH gene, were synthesized and each was added at a concentration of 10 nM. As template RNA, total RNA extracted from Hela cells was used in an amount of 100 ng, 10 ng, 1 ng or 100 pg. Then, distilled water was added to the mixture to a final volume of 50 $\mu\ell$. Real-time reverse transcription PCR was performed using Exicycler 96 Real-Time Quantitative Thermal Block (Bioneer, Korea) under the following conditions: reverse transcription at 50°C for 15 minutes, and then pre-denaturation at 95°C for 5 min, and then 45 cycles, each cycle consisting of denaturation at 95°C for 5 sec, annealing at 60°C for 5 sec, extension at 72°C for 6 sec, and scanning for detection of fluorescence.

[0095] As a result, it was shown that PPi and PPase did not influence the real-time reverse transcription PCR, and a standard graph having a slope of -3.1 and a linear R^2 value of 0.9994 was plotted (FIG. 3).

Example 4: Detection of hepatitis C virus RNA by hot-start reverse transcription reaction

[0096] To perform real-time reverse transcription PCR, template RNA was prepared. Specifically, a Hepatitis C virus gene was synthesized by a gene synthesis method (see Biochem. Biophys, Res. Commun 1998, 248, 200-203), and a portion thereof was cloned into a pGEM-T-Easy vector (Cat. A1360, Promega, USA). Specifically, 5 $\mu\ell$ of 2X rapid ligation buffer (Promega, USA), 1 $\mu\ell$, of T-easy vector (Promega, USA), 1 $\mu\ell$, of T4 DNA ligase (Promega, USA), and 3 $\mu\ell$ (8 ng) of the synthesized gene were placed and mixed in the same tube, and then incubated to stand at 37°C for 1 hour. Next, 5 $\mu\ell$, of the incubated reaction solution was added to 50 $\mu\ell$ of *E. coli* competent cells, kept on ice for 40 minutes, incubated at 42°C for 40 seconds, and then kept on ice for 2 minutes. The reaction solution was seeded into an LB plate containing ampicillin, isopropyl 1-thio-β-D-galactoside (IPTG) and X-gal, and was then incubated at 37°C for 16 hours. After incubation, white colonies were taken, incubated in LB liquid medium for 16 hours and then centrifuged. The supernatant was removed, and plasmid DNA was extracted from the pellet using an AccuPrep Plasmid Prep kit (Bioneer, Korea). When the plasmid DNA had a purity of 1.8-2.0 as measured by an UV spectrophotometer (Shimazu, Japan), the plasmid DNA was transcribed into RNA using a MAXIscript In vitro transcription kit (Ambion, USA. After transcription, when the RNA had a purity of 1.8-2.0 as measured by an UV spectrophotometer, it was used as template RNA in a subsequent real-time reverse transcription PCR. The copy number of the RNA was calculated using the following equation 1:

Equation 1

$$6.02 \times 10^{23} \times \text{concentration (g/m}\ell\text{, measured by UV spectrophotometer)} / (3015 + 150) \times 340$$

wherein 3015 represents the size of T-vector (3015 bp), and 150 represents the size of hepatitis C virus template RNA (150 bp). After calculating the copy number of the template RNA was serially 10-fold diluted with 1× TE buffer (10 mM Tris-HCl, pH 8.0, 0.1 mM EDTA) and was stored at -70°C until use. Using the constructed RNA as a template, real-time reverse transcription PCR was performed using 30 nM of the hepatitis C virus forward primer 5'-ACCGGGTCCTTTCTT-GGAT-3' (SEQ ID NO: 6), the reverse primer 5'-CCCTATCAGGCAGTACCACA-3' (SEQ ID NO: 7) and the probe [5'FAM] -CGTGCCCCCGCRAGACTGCT-[3'BHQ1] (SEQ ID NO: 8). The reactants used in the real-time reverse transcription PCR were the same as described in Example 3, and the template RNA with a copy number of 10^{10}, 10^9, 10^8, 10^7, 10^6, 10^5, 10^4, 10^3, 10^2 or 10 was added. The reaction was performed in the same manner as described in Example 3, except for the primer, the probe and the template RNA.

[0097] As a result, the template RNA with a copy number ranging from 10^{10} to 10 could be detected and a standard graph of the standard template real-time reverse transcription PCR that was repeated twice showed a slope of -3.21 to -3.73 and a linear R^2 value of 0.995-0.998 (FIG. 4). Herein, R^2 is a correlation ship that indicates the linearity of the plotted standard graph of the real-time PCR, and a R^2 closer to 1 (closer to a straight line) indicates that PCR was more accurately performed.

Example 5: Examination of the effect of PPi and PPase on inhibition of non-specific reaction in real-time reverse transcription PCR

[0098] In order to examine the effect of PPi and PPase on the non-specific reaction of purified RNA in an actual clinical sample, 1 μg of total RNA extracted from Hela cells in addition to the target template RNA was added to the same primers, probe and real-time reverse transcription PCR solution as described in Example 4. As a control, a reaction solution having no hot-start reverse transcription function (that is, having no PPi and PPase) was used. In order to examine the inhibition of non-specific reactions, 1 μg of total RNA extracted from human cells was added to and reacted with each of a hot-start reverse transcription reaction solution and a reaction solution having no hot-start reverse transcription function. This Example was performed in the same manner as described in Example 4, except that the extracted RNA was added.

[0099] As a result, it was found that, when the hot-start reverse transcription reaction was performed in the presence of PPi and PPase, non-specific reactions by RNAs other than the target RNA were inhibited, and RNAs other than the target RNA did not result in a decrease in reaction efficiency and changes in sensitivity and detection ability. However, it was shown that, in the hot-start reverse transcription that was performed in the absence of PPi and PPase, non-specific reactions were caused by the added human cell RNA, and reaction efficiency, detection ability and sensitivity significantly decreased (FIG. 5a). The product from the real-time reverse transcription PCR was analyzed by electrophoresis on 2% agarose gel, and as a result, it was found that, in case of the product not subjected to the hot-start reverse transcription reaction, non-specific reactions more actively occurred due to the total RNA extracted from the human cells (FIG. 5b). Thus, it can be seen that problems such as a decrease in reaction efficiency in non-specific reactions in reverse transcription PCR reactions can be solved by a hot-start reverse transcription reaction in the presence of PPi and PPase.

Example 6: Comparison of inhibition of non-specific reactions between use of hot-start reverse transcription reaction and use of hot-start PCR in real-time reverse transcription PCR

[0100] In order to examine which of a hot-start reverse transcription reaction and a hot-start PCR is more effective in reaction specificity and sensitivity in real-time reverse transcription PCR, the following experiment was performed. In addition to the same primers, probe and real-time reverse transcription PCR reaction solution as described in Example 5, target template RNA and 1 μg of total RNA extracted from human cells were used. A reaction solution having no hot-start reverse transcription function (that is, having no PPi and PPase) was used in a hot-start PCR reaction mix comprising Taq antibody. To examine the effect of the hot-start reverse transcription reaction on the inhibition of non-specific reactions, PPi and PPase were added to a hot-start PCR reaction mix comprising Taq antibody, and a hot-start reverse transcription reaction and a hot-start PCR were simultaneously used.

[0101] As a result, it was found that, in reverse transcription and PCR, non-specific reactions could not be inhibited by the hot-start PCR reaction alone, and when the hot-start reverse transcription reaction was performed in the presence of PPi and PPase, non-specific reactions for a large amount of RNA could be inhibited. Also, it was shown that the hot-start PCR reaction employing Taq antibody inhibited non-specific PCR reactions at low temperature, but could not inhibit non-specific reactions in the reverse transcription reaction. Thus, it can be seen that the hot-start reverse transcription reaction is required to eliminate non-specific reactions in the reverse transcription reaction and the PCR reaction. In both the reverse transcription reaction and the PCR reaction, non-specific reaction products decreased in the PCR reaction performed using Taq antibody (B of FIG. 6b) compared to those in the reaction having no hot start function (B of FIG. 5b), non-specific reactions still occurred, and thus it was difficult to detect disease virus such as the target hepatitis C virus DNA having a copy number of 100 or less in samples containing large amounts of human RNAs derived from human cells (B of FIG. 6a). On the other hand, in the reaction comprising PPi and PPase, non-specific reactions by RNA derived from human cells did not occur (D of FIG. 6b), the target hepatitis C virus DNA with a copy number of 10 or more could be detected with high density (D of FIG. 6a).

Example 7: Examination of standard template real-time reverse transcription PCR using a dry-type reverse transcription/PCR composition

[0102] In order to examine the thermal stability of a dry material of reverse transcription/PCR mixture solution, a PCR mixture solution containing PPi and PPase as described in Example 4 was prepared and dried, and real-time reverse transcription PCR was performed in an Exicycler Quantitative Thermal Block (Bioneer, Korea) using the dried mixture. Specifically, 5 $\mu\ell$ of 10× buffer, 3 U of Taq DNA polymerase, 5 $\mu\ell$ of dNTP 20 mM, PPi, PPase, stabilizer and the like were introduced into a single tube, and distilled water was added to a total volume of 25 $\mu\ell$. The mixture solution was dispensed into the tube of Exicycler Quantitative Thermal Block (Bioneer, Korea) and dried using SuperCentra (Bioneer, Korea) for 50-60 minutes. Next, the primers and probe used in Example 4 were mixed with each other to a total volume of 5 $\mu\ell$ and added to the dried mixture, followed by drying for 30 minutes. To the dried mixture, the template RNA as

used in Example 6 was added, and distilled water was added thereto to a total volume 50 $\mu\ell$, and the resulting solution was sufficiently mixed so that the dried material was easily dissolved. Real-time reverse transcription PCR was performed in the same manner as described in Example 4, except that Exicycler Quantitative Thermal Block (Bioneer, Korea) was used, a negative control (blank sample having no template RNA) was also reacted, and a template RNA with a copy number of 10^6, 10^5, 10^4, 10^3, 10^2 or 10 was used.

[0103] As a result, like Example 4, the template RNA with a copy number of 10 or more could be detected, and when the standard graph of the standard template real-time reverse transcription PCR was plotted, it showed a slope of -0.2932 and an R^2 value of 0.9991. The above results suggest that the dried mixture showed the same performance as a solution-state mixture in real-time reverse transcription PCR (FIG. 7).

Example 8: Test for stability of dried reverse transcription/PCR reaction composition at various storage periods

[0104] Using the same composition and method as described in Example 7, a PCR mixture containing PPi and PPase was prepared, and then stored at 50°C at 1-day intervals for 8 days. Then, the dried PCR composition according to each storage period was treated using Exicycler96 Quantitative Thermal Block, and the template RNA was used with a copy number of 10^6, 10^5, 10^4 or 10^3, and real-time reverse transcription PCR was performed under the same conditions as described in Example 7. This is a method making it possible to predict the period of maintenance of performance of the dried composition at -20°C (actual recommended storage temperature) by an accelerated test. When the composition was stored at 40°C for 1 day, it is regarded to be stored at - 20°C for about 128 days. Specifically, the dried composition was prepared in an amount corresponding to 6 tests, and was dried. Immediately after drying, a portion of the dried mixture was prepared in an amount corresponding to 5 tests under the same conditions as described in Example 7. Then, real-time reverse transcription PCR was performed in the same manner as described in Example 4, except that the template RNA was used with a copy number of 10^6, 10^5, 10^4 or 10^3, thereby obtaining the results of a control. The dried composition was stored in an incubator at 50°C, and the amount required for the reaction was taken at one-day intervals and subjected to real-time reverse transcription PCR. At this time, the reaction was performed using four copy numbers (ranging from 10^3 to 10^6) of RNA. The dried composition stored for each period was subjected to real-time reverse transcription PCR reaction using Exicycler96 Quantitative Thermal Block (Bioneer, Korea), and among the results of the reaction with dried mixtures classified by storage days, the slope value and the R^2 value were compared with those of the control group, thereby comparing the performance of the dried mixture immediately after preparation with that of the dried mixture stored at 50°C for each period.

[0105] As a result, the liquid mixture (control group) stored at 50°C at 1-day intervals for 5 days showed a slope of -0.345 and an R^2 value of 0.9922, and the dried composition stored at 50°C showed a slope of -0.31 to -0.34 and an R^2 value of 0.995-0.998. In other words, the results after 5 days of storage at 50°C were equal to the results immediately after drying, and such results were substantially similar to the results obtained after storage for about 640 days at -20°C (FIGS. 8a and 8b).

Example 9: Inhibition of non-specific binding of primers by hot-start reverse transcription PCR reaction

[0106] To induce the non-specific binding of primers, the nucleotide sequence of the reverse primer among the primers and probe used in Example 4 was synthesized as shown in Table 2 below.

[0107] Reverser primer 5'-CCCTATCAGGCAGTACCACA-3' (SEQ ID NO: 7)

Table 2

	Nucleotide sequence
Reverse primer	5'-CCCTATCAGGCAGTACCACA-3'(SEQ ID NO: 7)
Reverse primer having 6 mismatch nucleotides at the 5' end	5'-**GGGGGG**CAGGCAGTACCACA-3'(SEQ ID NO: 9)
Primer having 6 mismatch nucleotides in the middle of the nucleotide sequence	5'-CCCTA**TTTTTTT**GTACCACA-3'(SEQ ID NO: 10)

[0108] In order to examine the effect of non-specific primers, each of a reverse primer exactly matching the target template RNA, a reverse primer (SEQ ID NO: 9) having 6 mismatch nucleotides at the 5' end, and a primer (SEQ ID NO: 10) having 6 mismatch nucleotides in the middle of the nucleotide sequence was used. As a control, a reaction solution having no hot-start reverse transcription function (that is, having no PPi and PPase) was used. In order to examine whether non-specific primers cause false positive results, whether each reverse primer cause non-specific reactions was examined using a hot-start reverse transcription reaction solution and a reaction solution having no hot-

start reverse transcription function. The procedure of Example 4 was repeated, except that the mismatch reverse primer was used and only a template RNA with a copy number of 10^4 was used.

[0109] FIG. 9a shows the results of performing a real-time reverse transcription PCR using the primer having 6 mismatch nucleotides at the 5' end, and FIG. 9b shows performing a real-time reverse transcription PCR using the primer having 6 mismatch nucleotides in the nucleotide sequence. In FIG. 9, "A" indicates the results of performing the reaction using the reaction solution having no host-start reverse transcription function, and "B" indicates the results of performing the reaction using the hot-start reverse transcription PCR reaction mixture.

[0110] As a result, in the case of the hot-start reverse transcription performed in the presence of PPi and PPase, the amplification product was detected in the reverse primer exactly matching the template RNA, but was not detected in the reverse primer having mismatch nucleotides. However, in the case of the hot-start reverse transcription performed in the absence of PPi and PPase, the amplification product was detected even in the reverse primer having mismatch nucleotides. This suggests that enzymatic reactions occur due to the non-specific reaction of the reverse primer at a temperature lower than the reaction temperature (FIGS. 9a and 9b). Thus, it can be seen that problems such as false positive results caused by non-specific reactions in the reverse transcription PCR reaction can be solved by the hot-start reverse transcription reaction in the presence of PPi and PPase.

Example 10: Analysis of single-nucleotide polymorphism by hot-start reverse transcription PCR reaction

[0111] To analyze the single-nucleotide polymorphism of the target RNA using the hot-start reverse transcription PCR reaction of the present invention, a reverse primer was designed such that it contains a point mutation. The nucleotide sequence of the reverse primer among the primers and probe used in Example 4 was synthesized as shown in Table 3 below.

Table 3

	Nucleotide sequences
Reverse primer	5'-CCCTATCAGGCAGTACCACA-3'(SEQ ID NO: 7)
Reverse primer having a single mismatch nucleotide	5'-CCCTATCAGGCAGT**C**CCACA-3'(SEQ ID NO: 11)

[0112] For analysis of single-nucleotide polymorphism, a reverse primer exactly matching the template RNA and a reverse primer having a single mismatch nucleotide were used. As a control, a reaction solution having no hot-start reverse transcription function (that is, having no PPi and PPase) was used. In order to examine whether the analysis of single-nucleotide polymorphism is possible, a reaction for each reverse primer was analyzed using a hot-start reverse transcription reaction solution and a reaction solution having no hot-start reverse transcription function. The analysis was performed in the same manner as described in Example 4, except that a reverse primer having a mismatch nucleotide was used and a template RNA with a copy number of 10^4 was used. FIG. 10a shows the results of the hot-start reverse transcription PCR reaction, and FIG. 10b shows the results of performing the reaction using the reaction solution having no hot-start reverse transcription function. In FIG. 10, "A" indicates the results obtained using the primer having an exactly matching nucleotide sequence, and "B" shows the results obtained using the primer having a single mismatching nucleotide.

[0113] As a result, it was found that, when the hot-start reverse transcription reaction was performed in the presence of PPi and PPase, the results obtained using the primer exactly matching the template RNA did differ in PCR efficiency from the results obtained using the reverse primer having a single mismatch nucleotide. However, the hot-start reverse transcription reaction performed in the absence of PPi and PPase was detected in the same cycle without difference between the exactly matching reverse primer and the mismatch reverse primer. This suggests that the hot-start reverse transcription reaction makes it possible to analyze single-nucleotide polymorphism using a real-time PCR method, but in a reaction performed in the absence of a hot-start reverse transcription reaction mixture, it is difficult to analyze single-nucleotide polymorphism (FIGS. 10a and 10b). Thus, it was found that the hot-start reverse transcription reaction enables single-nucleotide analysis for the template RNA and can be used in various fields.

Example 11: Inhibition of RNA-RNA self-priming by hot-start reverse transcription reaction

[0114] The effect of the hot-start reverse transcription PCR reaction of the present invention on the inhibition of non-specific reactions resulting from self-priming of a single-stranded nucleic acid contained in an extracted nucleic acid was

examined.

[0115] Total RNA was extracted from 10^6 Hela cells using AccuZole Total RNA Extraction solution (Bioneer, Korea), and a final volume of 50 μl was obtained using DEPC-D.W.

[0116] The extracted RNA was quantified using NanoDrop 2000 (Thermo Scientific, USA), and 2 μg of the RNA was used. A reverse transcription reaction was performed using a reaction solution having no hot-start reverse transcription function (that is, having no PPi and PPase) as a control. A real-time PCR reaction was performed using AccuPower Dualstar qPCR Premix kit (Bioneer, Korea). The real-time PCR reaction was performed using the forward and reverse primers and probe used in Example 3. The reverse transcription reaction was performed using the primer 5'-TTTTTTTTTTTTTTTTTT-3' (SEQ ID NO: 12).

[0117] In order to examine whether the extracted RNA contains genomic DNA, a reverse transcription reaction was performed using a reaction solution having no hot-start reverse transcription function (that is, having no PPi and PPase) as a control, and the extracted total RNA as a template was subjected to real-time PCR using AccuPower Dualstar qPCR Premix (Bioneer, Korea) without reverse transcription reaction.

[0118] In FIG. 11, A shows the results of performing a real-time PCR reaction using RNA alone without reverse transcription reaction; and B shows the results of performing a reverse transcription reaction using the primer and then performing a real-time PCR reaction. B-A in FIG. 11 indicates the result of performing a hot-start reverse transcription reaction, and B-B indicates the result of performing a reaction using a reaction solution having no hot-start reverse transcription function.

[0119] FIG. 11C shows the results of performing a reverse transcription reaction using RNA alone in a reaction solution having no hot-start reverse transcription function and then performing a real-time PCR reaction. FIG. 11D shows the results of performing a reverse transcription reaction using RNA alone in a hot-start reverse transcription reaction mixture and then performing a real-time PCR reaction.

[0120] As a result, it was found that no detection was found in the absence of reverse transcription reaction, suggesting that the extracted total RNA contained no genomic DNA.

[0121] As a control, a reaction solution having no hot-start function (that is, having no PPi and PPase), and a reverse transcription reaction was performed using total RNA alone in the reaction solution without adding a primer. The reverse transcription reaction was performed at 50°C for 1 hour and 95°C for 5 minutes. A real-time PCR reaction was performed 5 μℓ of the reverse transcription reaction product as a template.

[0122] As a result, in the reaction solution having no hot-start function (that is, having no PPi and PPase), detection was found at 37 C(t) by self-priming, and in the hot-start reverse transcription reaction solution, detection was found at 41 C(t) (FIG. 11). Thus, it was found that non-specific reactions by self-priming were more inhibited in the hot-start reverse transcription reaction solution compared to those in the reaction solution having no hot-start function (that is, having no (PPi and PPase).

Example 12: Effect of RNA termination on inhibition of non-specific reverse transcription PCR caused by RNA self-priming

(1) Termination reaction of RNA fragment with poly (A) polymerase and 3'-deoxyadenosine 5'-triphosphate (3'-dATP)

[0123] Using an RNA extraction kit as described in Example 11, RNA was extracted from human HeLa C cells. The 3' end of the extracted RNA was terminated with poly(A) polymerase and 3'-deoxyadenosine 5'-triphosphate (3'-dATP) (C and D).

[0124] To remove the remaining 3'-deoxyadenosine 5'-triphosphate (3'-dATP), the terminated RNA was purified using AccuPrep PCR Purification kit (Bioneer, Korea). For comparison, RNA (A and B) which was not terminated at the 3' end were purified in the same manner as above. To examine the effect of a hot-start reverse transcription PCR, a PCR reaction was performed in each of the hot-start reverse transcription PCR reaction solution (B and D) and the PCR reaction solution having no hot-start function (A and C).

[0125] 5 μg of RNA was used in each PCR reaction. A general PCR reaction was performed using AccuPower RocketScript RT Premix (Bioneer, Korea), and the hot-start PCR reaction was performed using AccuPower RocketScript RT Premix (Bioneer, Korea), which comprises pyrophosphate and phosphatase added thereto and distilled water added thereto to a final volume of 20 μl. To examine of RNA self-priming, any primer required for reverse transcription PCR was not added.

[0126] The reverse transcription PCR was performed using Mygenie 96 Gradient Thermal Block (Bioneer, Korea), and to examine the effect of RNA self-priming during mixing of reactants, the PCR reaction mixture was incubated at 25°C for 20 minutes.

[0127] To examine whether the reverse transcription PCR is caused by RNA self-priming, real-time reverse transcription PCR was performed using each reverse transcription PCR reaction mixture as a template, AccuPower Dualstar qPCR Premix (Bioneer, Korea) and the human GAPDH-targeting primers and probe used in Example 3.

[0128] The real-time PCR was performed using Exicycler 96 Real-Time Quantitative Thermal Block (Bioneer, Korea) under the following conditions: pre-denaturation at 95°C for 5 min, and then 45 cycles, each cycle consisting of denaturation at 95°C for 5 sec, annealing and extension at 60°C for 5 sec, and scanning for fluorescence detection.

[0129] The results are shown in FIG. 12. As can be seen therein, in the case of the non-terminated RNAs (A and B), the human GAPDH gene was detected, suggesting that cDNA was synthesized even when no primer was added. This result indicates that cDNA was synthesized by self-priming and amplified in PCR.

[0130] Also, the PCR amplification resulting from the non-specific cDNA synthesis could not be perfectly prevented by the hot-start reverse transcription PCR reaction alone, and the reverse transcription PCR reaction caused by RNA self-priming could be partially inhibited, and thus the Ct value was reduced by about 5, indicating that the non-specific reaction could be inhibited by about 1/30.

[0131] However, it was shown that, in the samples comprising the RNA terminated at the 3' end (C and D), cDNA was not amplified up to 45 cycles in all the reverse transcription PCR reactions, indicating that no cDNA was synthesized. Above resulrs suggest that many non-specific reverse transcription PCR reactions occur during mixed reverse transcription PCR reaction mixture, and for this reason, many non-specific cDNAs are made. From the above results, it can be seen that undesired reactions cannot be inhibited by the hot-start reverse transcription PCR reaction alone. As a result, it can be seen that such non-specific reverse transcription PCR reactions resulting from RNA self-priming can be perfectly inhibited by terminating RNA using the termination reaction of the present invention.

[0132] In FIG. 12, "A" and "B" show the results obtained using non-terminated RNA, and C and D show the results obtained using the terminated RNA. Also, for "A" and "C", a general reverse transcription reaction was performed, and for "B" and "D", the hot-start reverse transcription reaction was performed.

Example 13: Increase in detection limit of cancer marker by RNA termination and hot-start reverse transcription PCR

[0133] The nucleotide sequence information of Homo sapiens Keratin 8 (KRT8; Accession No. NM_00125693) as a cancer marker was obtained from the National Center for Biotechnology Information (NCBI, USA). Using the RNA extraction kit as described in Example 11, RNA was extracted from human Hela C cells. Real-time reverse transcription PCR was performed using 850 ng, 85 ng, 8.5 ng, 0.85 ng or 0.085 ng of the extracted RNA as a template, 30 nM of the forward primer 5'-GGAGCAGATCAAGACCCTCA-3' (SEQ ID NO: 13), the reverse primer 5'-TTGTCCATGTTGCTTCGAGC-3' (SEQ ID NO: 14) and the probe [5'FAM]-TGCTGCTCCAGGAACCGTACCTTGT-[3'BHQ1] (SEQ ID NO: 15) in order to obtain a standard curve for performing quantitative analysis using a hot-start reverse transcription PCR reaction.

[0134] The results are shown in FIG. 13a.

[0135] In order to examine detection limit, 10,000, 1,000, 100 or 10 Hela cells were added to 1 ml of human serum, and RNA extraction and quantification were performed as described in Example 11.

[0136] As a result, it was found that the RNA concentrations were 57.5 ng/μl, 53.8 ng/μl, 52.5 ng/μl and 52.1 ng/μl. 500 ng of each extracted RNA was terminated according to the method of Example 12, and then quantified. To examine the effect of RNA termination on the detection limit, non-terminated RNA was used as a control. Real-time reverse transcription PCR was performed using 100 ng of each RNA in each of a reaction solution having no hot-start reverse transcription function (that is, having no PPi and PPase) and a hot-start reverse transcription PCR reaction solution. The real-time reverse transcription PCR was performed using Exicycler 96 Real-Time Quantitative Thermal Block (Bioneer, Korea) under the following conditions: reverse transcription reaction at 50°C for 15 minutes, and then pre-denaturation at 95°C for 5 min, followed by total 45 cycles, each cycle consisting of denaturation at 95°C for 5 sec, annealing at 60°C for 5 sec, extension at 72°C for 6 sec, and scanning for fluorescence detection.

[0137] As a result, in the case of the samples comprising the terminated RNA, the hot-start reverse transcription reaction solution was detected in the samples comprising 10 or more Hela cells added to 1 ml of human serum (see FIG. 13b). The reaction solutions having no hot start reverse transcription function was detected only in the sample comprising 100 or more Hela cells (FIG. 13c). In the case of the samples having no terminated RNA, the hot-start reverse transcription solution was detected in the samples comprising 100 or more Hela cells (FIG. 13d). In addition, the reaction having no hot-start reverse transcription function was detected in the samples comprising 1,000 or more Hela cells (FIG. 13e).

[0138] Thus, it can be seen that the detection limit was increased even by the hot-start reverse transcription reaction alone, but a better effect could be obtained when the hot-start reverse transcription reaction was performed together with RNA termination.

INDUSTRIAL APPLICABILITY

[0139] As described above, the composition for hot-start reverse transcription reaction and the composition for hot-start reverse transcription PCR according to the present invention can solve various problems occurring in conventional

hot-start reverse transcription reactions and can specifically detect the target RNA with high sensitivity by performing a reverse transcription reaction using PPi and PPase. Thus, the compositions of the present invention can provide a stable kit related to hot-start reverse transcription/hot-start reverse transcription PCR technologies, which can be effectively used as a high-sensitivity RNA diagnostic kit for high-sensitivity detection of RNA.

5

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<110>   Bioneer co. Ltd.

<120>   Compositions for hot start reverse transcription reaction or hot
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<151>   2012-03-09

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```

10

15

20

25

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40

45

50

55

Claims

1. A composition for hot-start reverse transcription reaction, which comprises an Mg^{2+} ion, four kinds of dNTPs, reverse

transcription polymerase, pyrophosphate (PPi), and pyrophosphatase (PPase).

2. The composition for hot-start reverse transcription reaction of claim 1, wherein the composition comprising pyrophosphate (PPi) at a concentration of 0.1-5 mM, and the pyrophosphatase in an amount of 0.005-0.25 U.

3. The composition for hot-start reverse transcription reaction of claim 1, further comprising one or more reverse transcription primers.

4. The composition for hot-start reverse transcription reaction of claim 1, wherein the composition is frozen or dried.

5. The composition for hot-start reverse transcription reaction of claim 1, further comprising template nucleic acid.

6. The composition for hot-start reverse transcription reaction of claim 5, wherein the template nucleic acid is an RNA.

7. The composition for hot-start reverse transcription reaction of claim 1, further comprising a dye which is not reactive with nucleic acid.

8. The composition for hot-start reverse transcription reaction of claim 1, further comprising at least one selected from the group consisting of a polyol, gelatin, bovine serum albumin, Thesit, and PEG-8000.

9. The composition for hot-start reverse transcription reaction of claim 5, wherein the template nucleic acid has a nucleic acid polymerization terminator bound to the 3' end thereof to prevent non-specific nucleic acid polymerization.

10. The composition for hot-start reverse transcription reaction of claim 9, wherein the nucleic acid polymerization terminator is a nucleic acid-like compound that is activated in the form of triphosphate capable of acting on nucleic acid polymerase and has groups other than a hydroxyl group at the 3' end.

11. The composition for hot-start reverse transcription reaction of claim 10, wherein the nucleic acid-like compound comprises at least one selected from among 2'3'-dideoxynucleoside 5'-triphosphate, 3'-deoxyadenosine 5'-triphosphate, 3'-azido-3'-deoxythymidine 5'-triphosphate, 1-β-d-Arabinofuranosylnucleoside 5'-Triphosphate, acyclo-guanosine triphosphate, 3'-amino-2'-deoxynucleoside 5'-triphosphate, and 3'-fluoro-3'-deoxynucleoside 5'-triphosphate.

12. A composition for hot-start reverse transcription PCR, which comprises an Mg^{2+} ion, four kinds of dNTPs, reverse transcription polymerase, pyrophosphate (PPi), and pyrophosphatase (PPase), and further comprises DNA polymerase.

13. The composition for hot-start reverse transcription PCR of claim 12, wherein the composition comprising pyrophosphate (PPi) at a concentration of 0.1-5 mM, and the pyrophosphatase in an amount of 0.005-0.25 U.

14. The composition for hot-start reverse transcription PCR of claim 12, further comprising one or more reverse transcription primers.

15. The composition for hot-start reverse transcription PCR of claim 12, further comprising probes.

16. The composition for hot-start reverse transcription PCR of claim 12, wherein the composition is frozen or dried.

17. The composition for hot-start reverse transcription PCR of claim 12, further comprising template nucleic acid.

18. The composition for hot-start reverse transcription PCR of claim 12, further comprising a fluorescent dye that binds to DNA.

19. The composition for hot-start reverse transcription PCR of claim 12, wherein the DNA polymerase comprises at least one selected from the group consisting of a polymerase having 5'->3' exonuclease activity, a polymerase having 3'->5' exonuclease activity, and a polymerase that does not have 5'->3' exonuclease activity and 3'->5' exonuclease activity.

20. The composition for hot-start reverse transcription PCR of claim 12, further comprising a dye which is non-reactive

with nucleic acid.

21. The composition for hot-start reverse transcription PCR of claim 17, wherein the template nucleic acid has a nucleic acid polymerization terminator bound to the 3' end thereof to prevent non-specific nucleic acid polymerization.

22. The composition for hot-start reverse transcription PCR of claim 21, wherein the nucleic acid polymerization terminator is a nucleic acid-like compound that is activated in the form of triphosphate capable of acting on nucleic acid polymerase and has groups other than a hydroxyl group at the 3' end.

23. The composition for hot-start reverse transcription PCR of claim 22, wherein the nucleic acid-like compound comprises at least one selected from among 2'3'-dideoxynucleoside 5'-triphosphate, 3'-deoxyadenosine 5'-triphosphate, 3'-azido-3'-deoxythymidine 5'-triphosphate, 1-β-d-Arabinofuranosylnucleoside 5'-Triphosphate, acyclo-guanosine triphosphate, 3'-amino-2'-deoxynucleoside 5'-triphosphate, and 3'-fluoro-3'-deoxynucleoside 5'-triphosphate.

24. The composition for hot-start reverse transcription PCR of claim 12, further comprising at least one selected from the group consisting of a polyol, gelatin, bovine serum albumin, Thesit, and PEG-8000.

25. The composition for hot-start reverse transcription PCR of claim 12, further comprising a substance that shows a hot-start PCR effect by binding to DNA polymerase or regulating the action of DNA polymerase.

26. The composition for hot-start reverse transcription PCR of claim 12, wherein the DNA polymerase shows hot-start PCR effect with a modified form thereof.

27. The composition for hot-start reverse transcription PCR of claim 26, wherein the substance is selected from the group consisting of an antibody, an aptamer and an affibody, which bind to DNA polymerase.

28. The composition for hot-start reverse transcription PCR of claim 12, wherein the composition is used in any one of a multiplex reverse transcription PCR reaction, a real-time reverse transcription PCR reaction, a real-time quantitative reverse transcription PCR reaction, and a multiplex real-time reverse transcription PCR reaction.

29. The composition for hot-start reverse transcription PCR of any one of claims 12 to 28, wherein the composition is a composition for diagnosis of a cancer.

30. A method of preparing a composition for a hot-start reverse transcription reaction, the method comprising introducing the composition for the hot-start reverse transcription reaction of any one of claims 1 to 11 into a single reaction tube.

31. A method of preparing a composition for a hot-start reverse transcription PCR, the method comprising introducing the composition for the hot-start reverse transcription PCR of any one of claims 12 to 28 into a single reaction tube.

32. A kit for hot-start reverse transcription reaction, which comprises the composition for hot-start reverse transcription reaction of any one of claims 1 to 11.

33. A kit for hot-start reverse transcription PCR, which comprises the composition for hot-start reverse transcription PCR of any one of claims 12 to 28.

34. A method of reverse transcription of template RNA, the method comprising the steps of:

mixing the composition for hot-start reverse transcription reaction of any one of claims 1 to 11 with a sample containing the template RNA to form a reaction mixture; and
performing a reaction so that the reaction mixture can be reverse-transcribed.

35. A method of amplifying nucleic acid, the method comprising the steps of:

mixing the composition for hot-start reverse transcription PCR of any one of claims 12 to 28 wit a sample containing template RNA to obtain a reaction mixture;
performing a reaction so as to amplify the reaction mixture to thereby obtain an amplification product; and
analyzing the amplification product.

36. The method of claim 35, wherein the PCR is selected from the group consisting of PCR, multiplex PCR, real-time PCR, and real-time quantitative PCR.

37. A composition of reverse transcription PCR for diagnosis of a cancer, which comprises a template nucleic acid that has a nucleic acid polymerization terminator bound to the 3' end to prevent non-specific nucleic acid polymerization, an Mg^{2+} ion, four kinds of dNTPs, reverse transcription polymerase, nucleic acid, primer for cDNA synthesis, pyrophosphate, pyrophosphatase, and a primer for amplification of a biomarker for diagnosis of a cancer.

Fig. 1

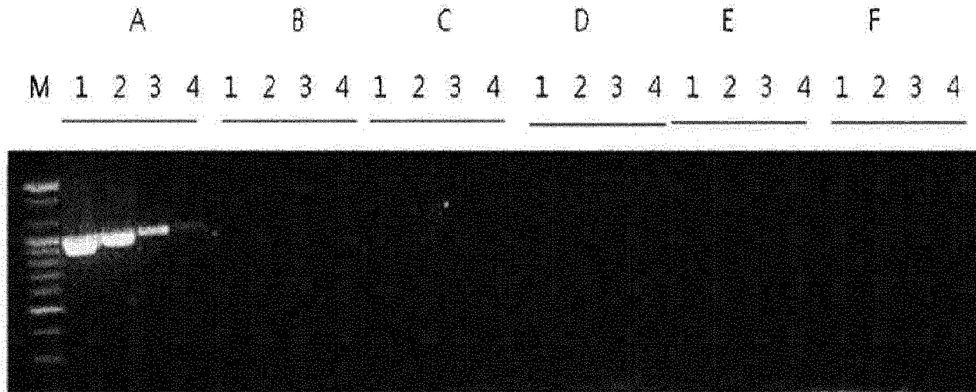


Fig. 2

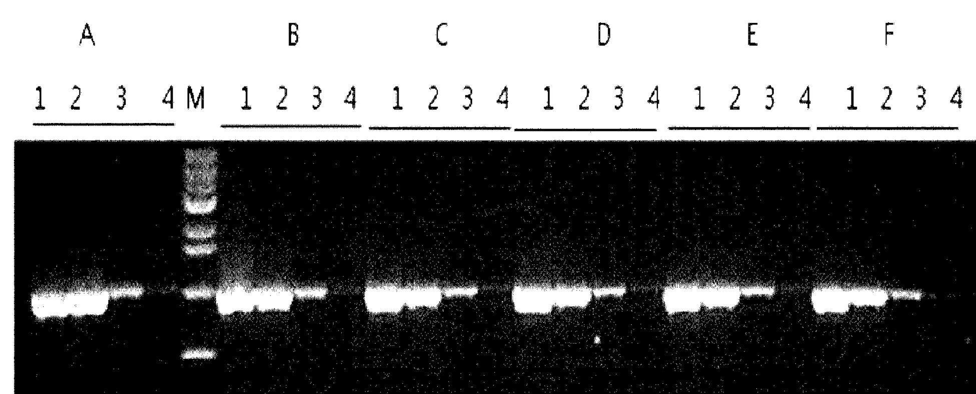


Fig. 3

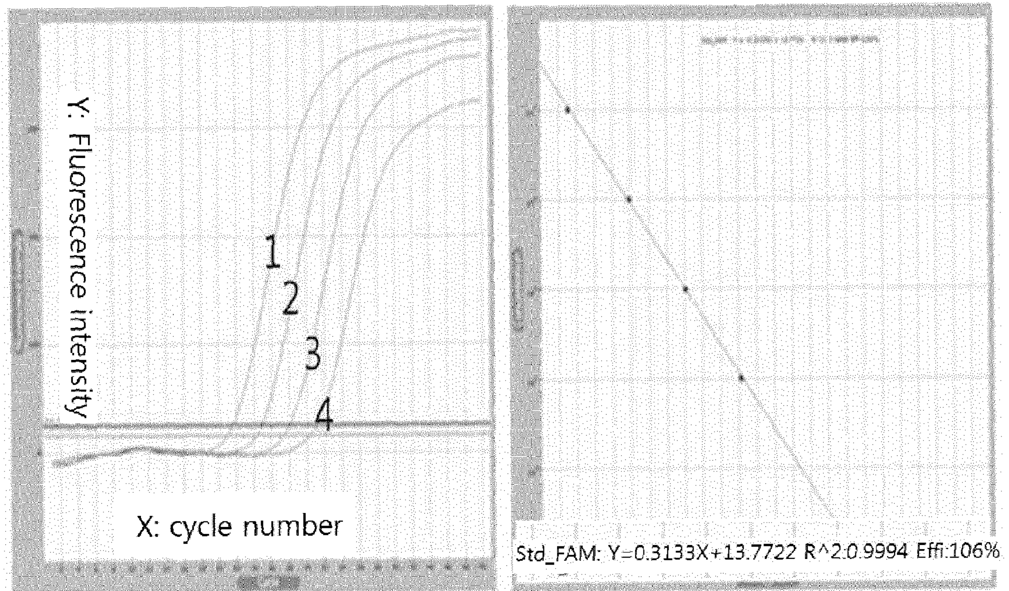


Fig. 4

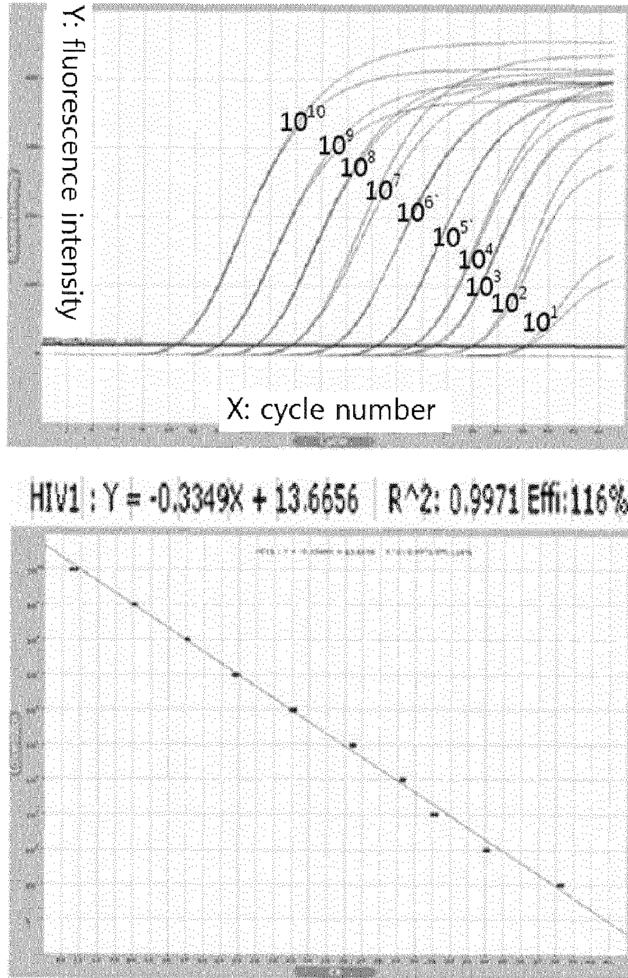


Fig. 5a

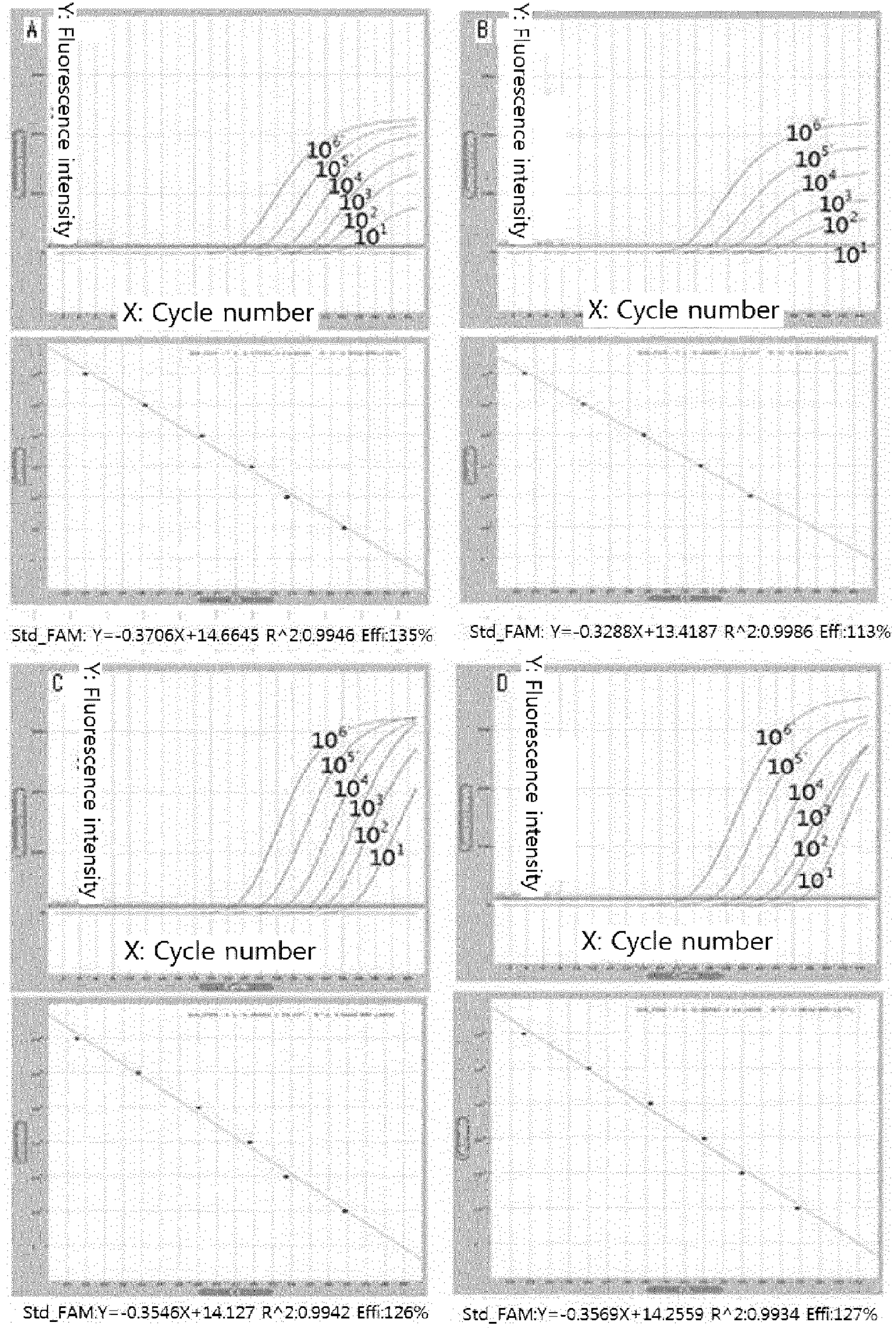


Fig. 5b

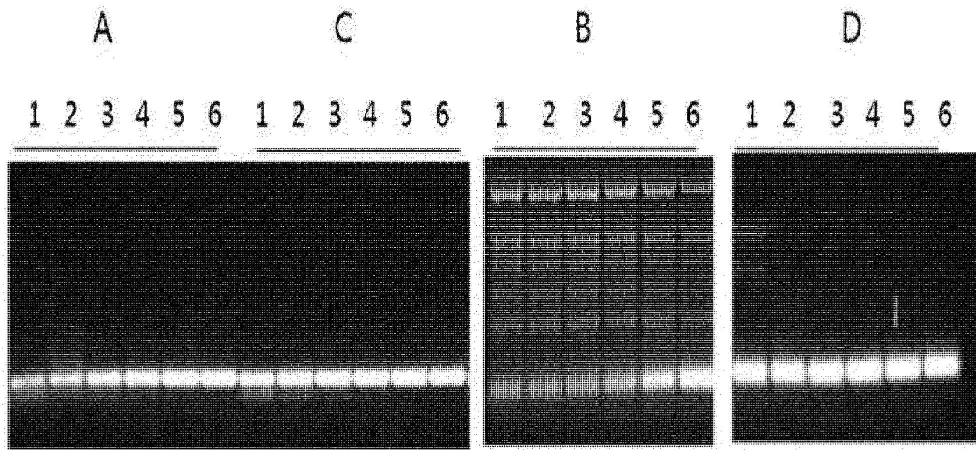


Fig. 6a

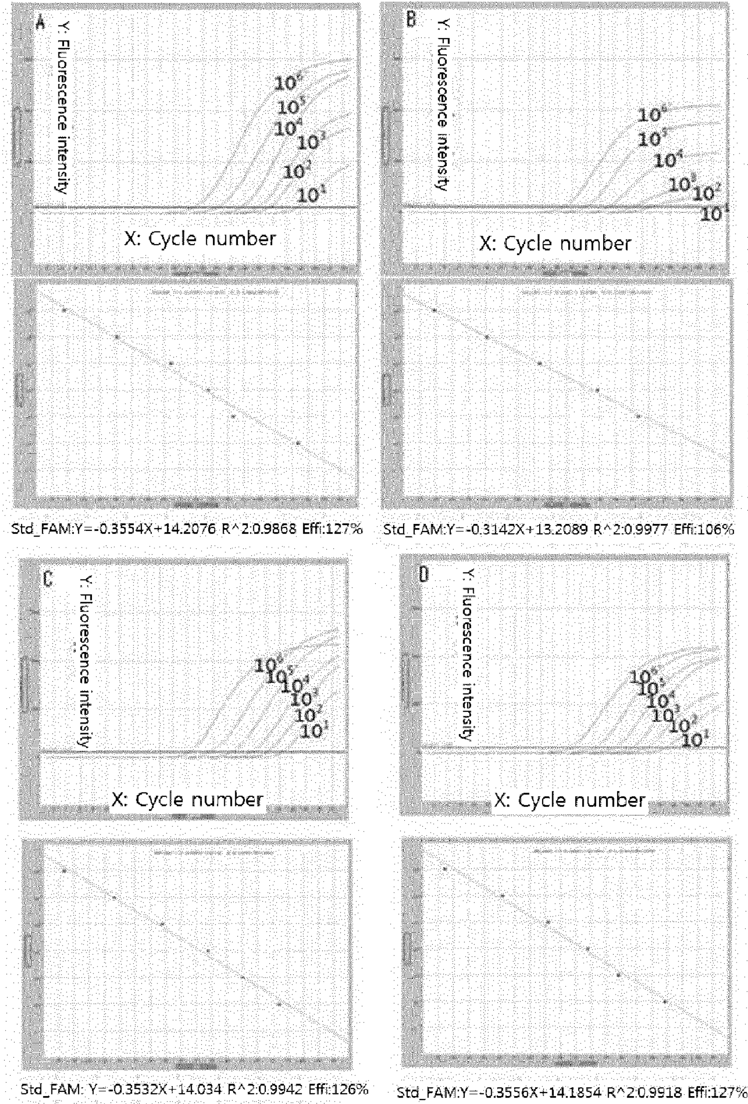


Fig. 6b

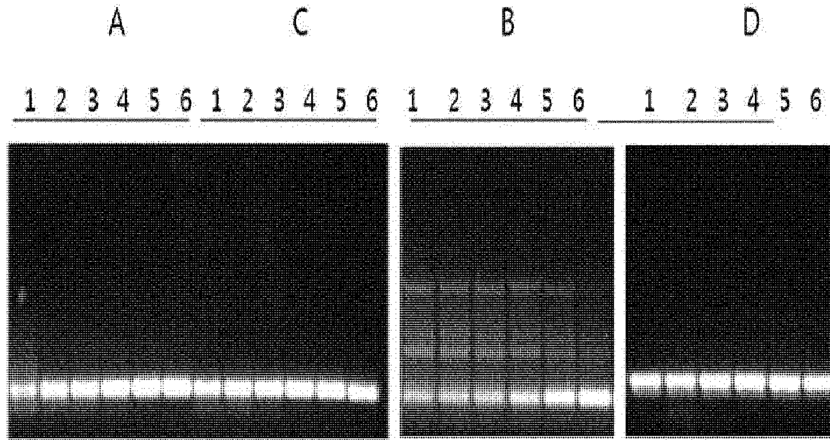


Fig. 7a

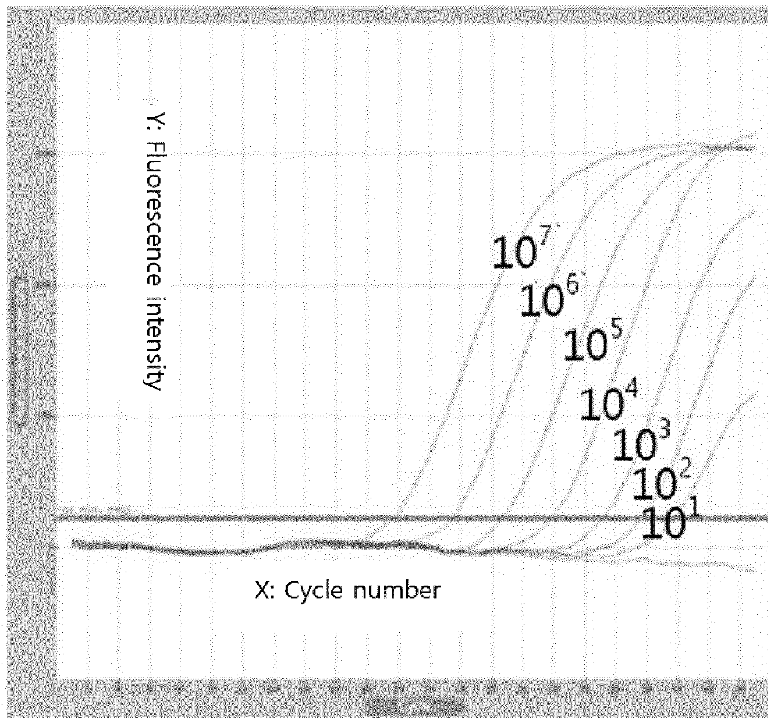
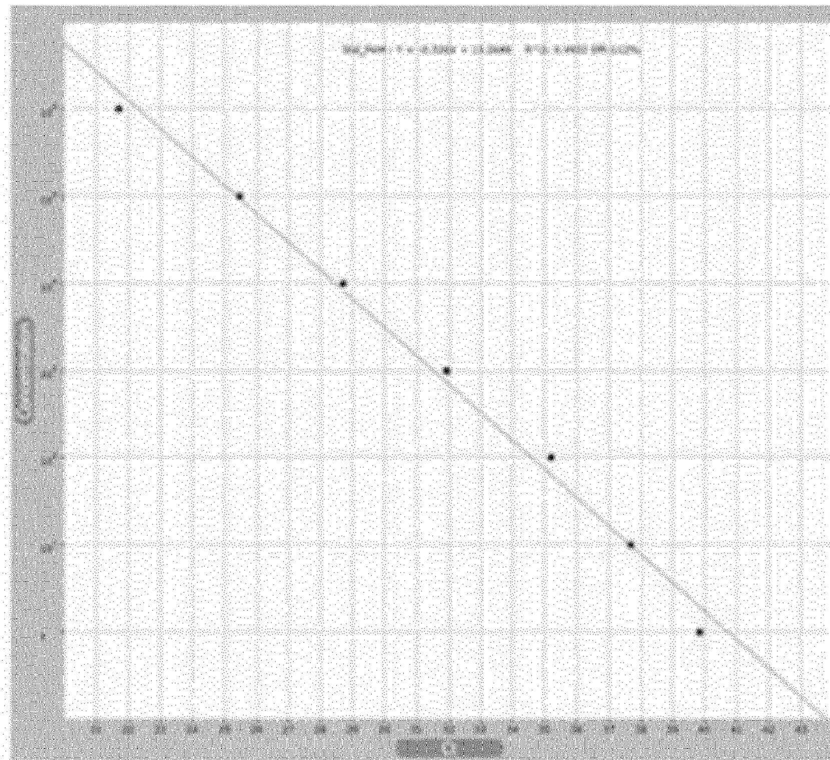


Fig. 7b



Std_FAM : Y = -0.326X + 13.2688 R^2: 0.9932 Effi:112%

Fig. 7c

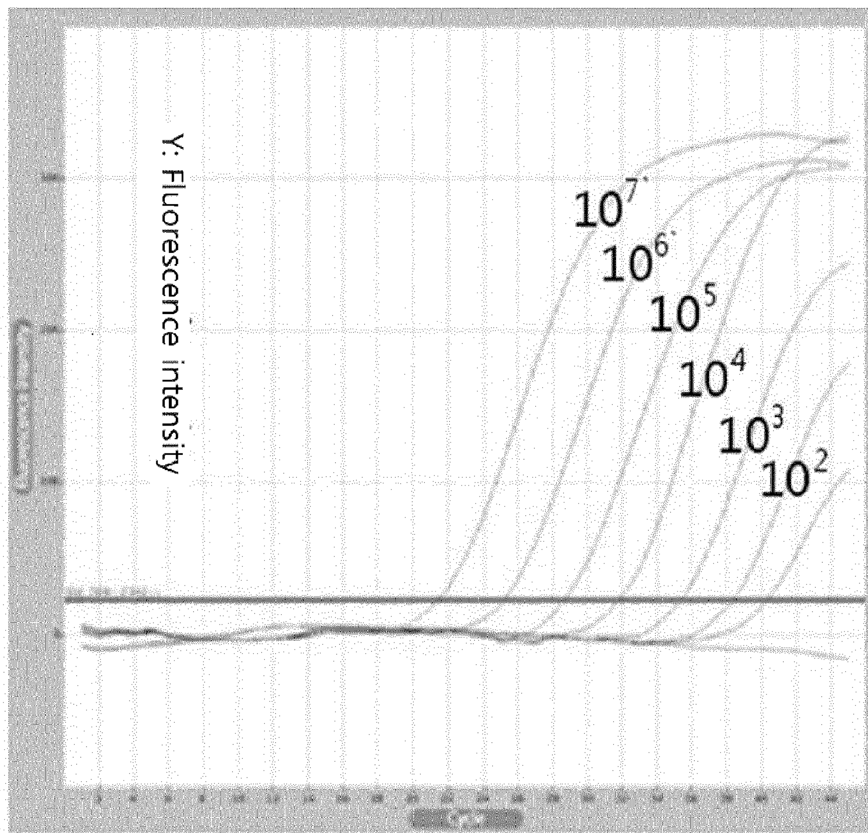
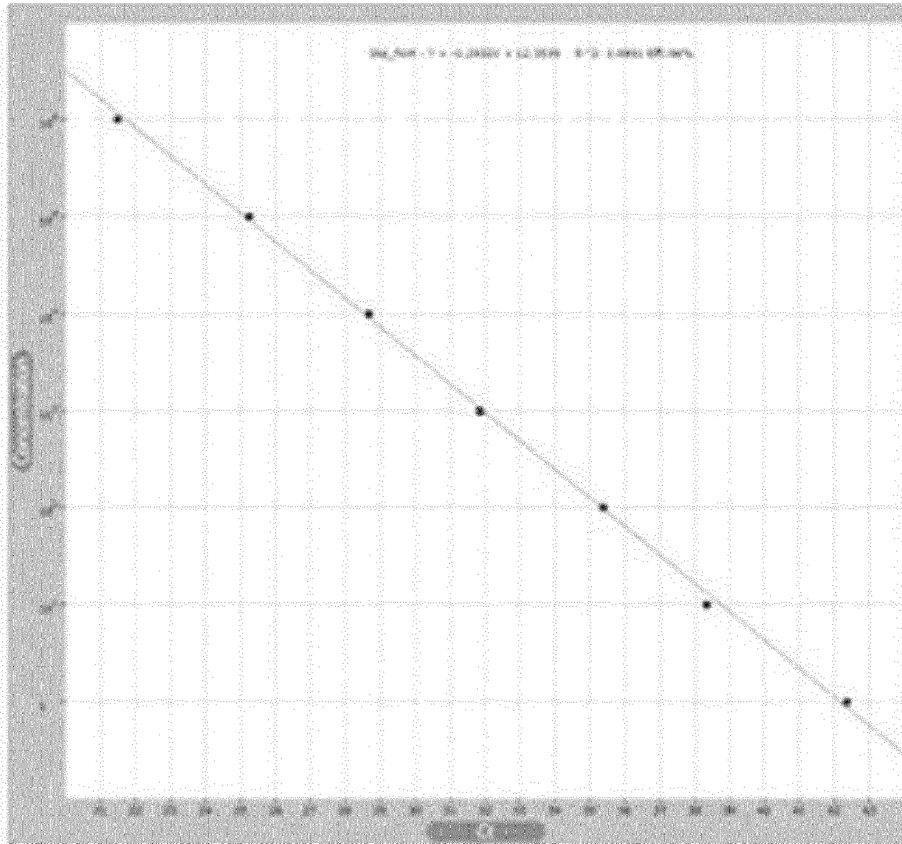


Fig. 7d



Std_FAM : Y = -0.2932X + 12.3539 R^2: 0.9991 Effi:96%

Fig. 8a

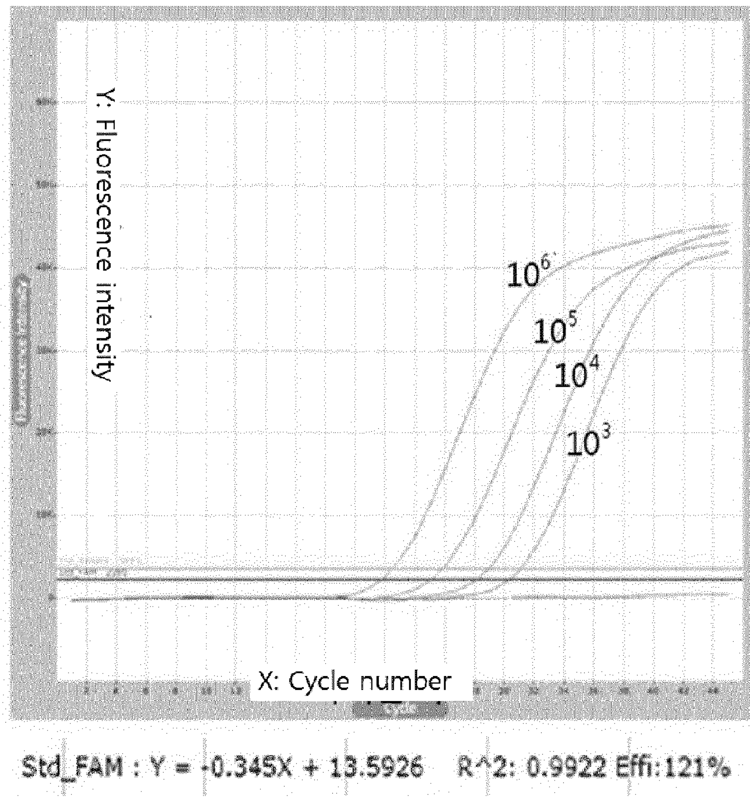
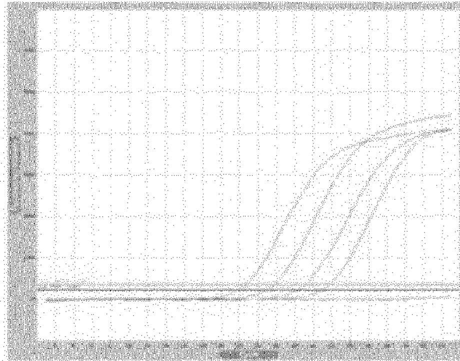
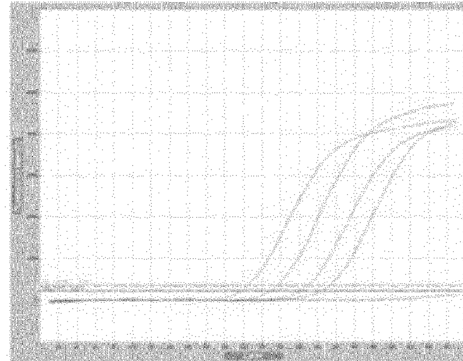


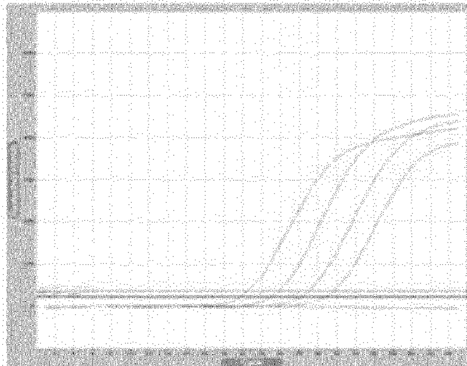
Fig. 8b



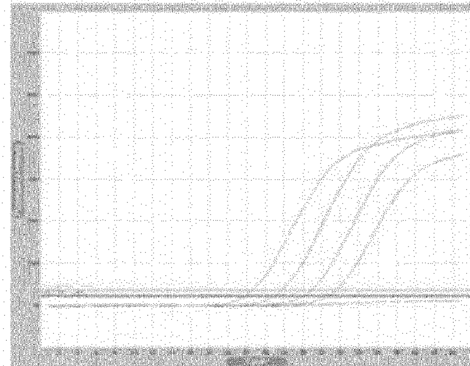
Std_FAM : Y = -0.3412X + 13.4991 R^2: 0.9941 Effi:119



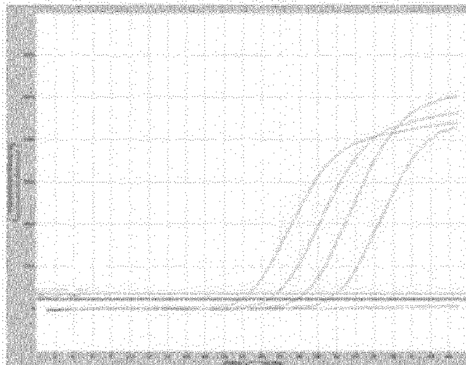
td_FAM : Y = -0.3302X + 13.2709 R^2: 0.9984 Effi:114%



Std_FAM : Y = -0.3321X + 13.2904 R^2: 0.9974 Effi:115%

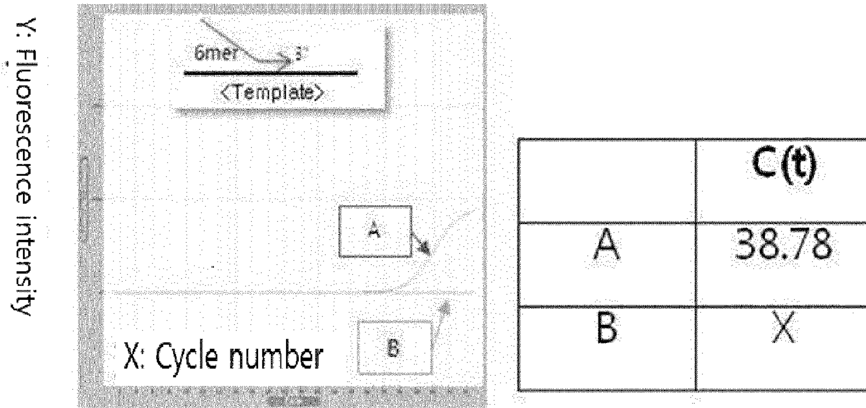


Std_FAM : Y = -0.3371X + 13.3986 R^2: 0.9976 Effi:117%



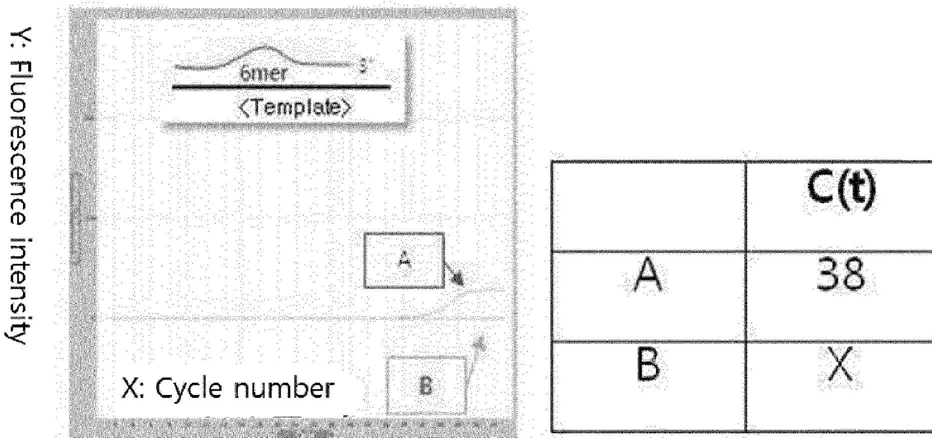
Std_FAM : Y = -0.3222X + 13.0028 R^2: 0.9986 Effi:110%

Fig. 9a



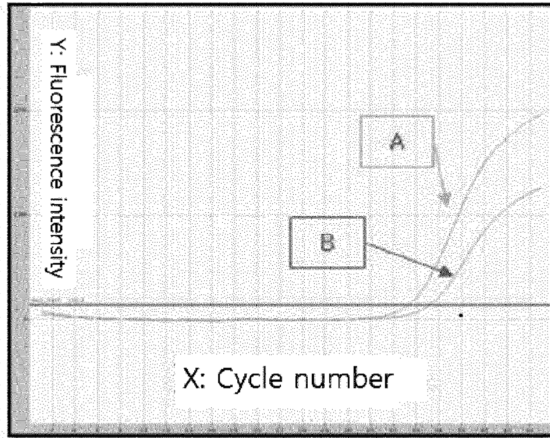
	C(t)
A	38.78
B	X

Fig. 9b



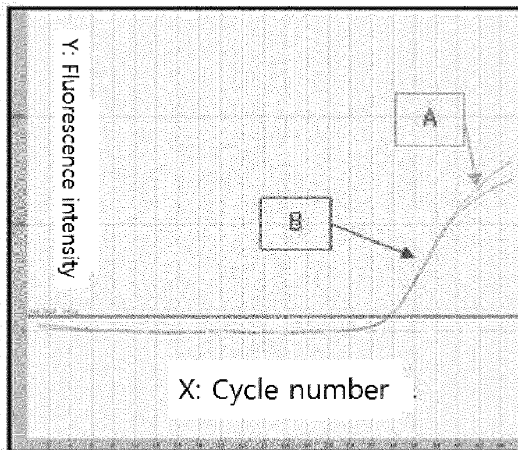
	C(t)
A	38
B	X

Fig. 10a



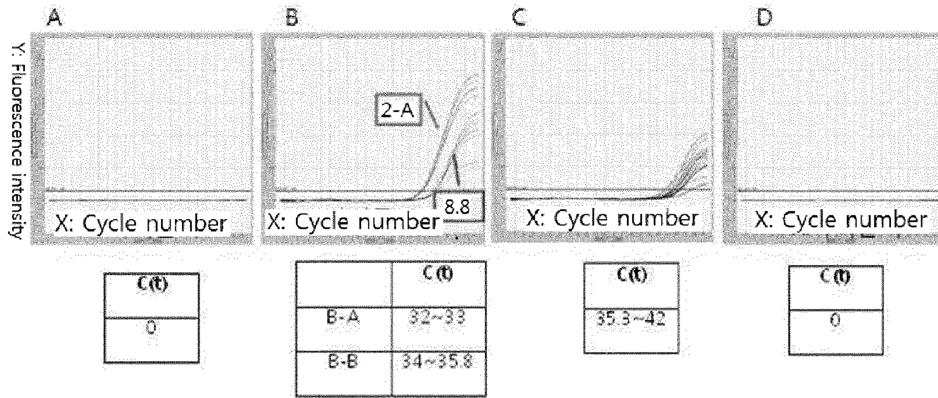
	C(t)
A	33.12
B	35.35

Fig. 10b



	C(t)
A	33.89
B	35.89

Fig. 11



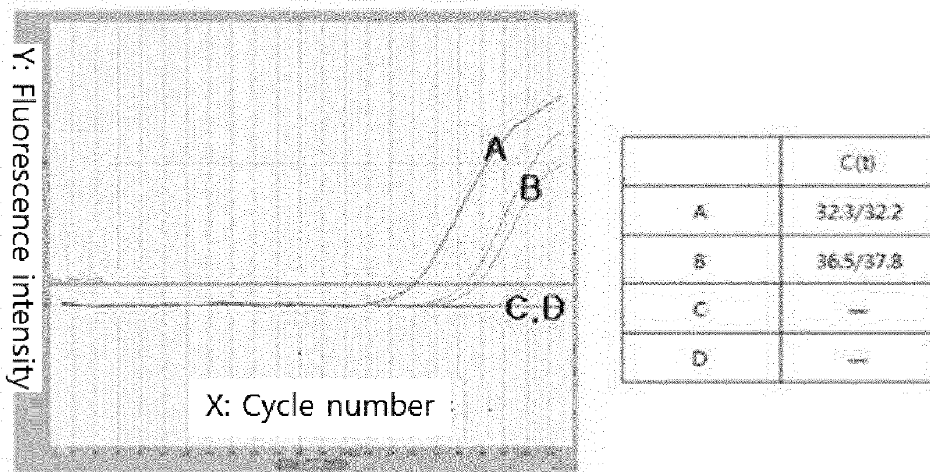
C(t)
0

	C(t)
B-A	32~33
B-B	34~35.8

C(t)
35.3~42

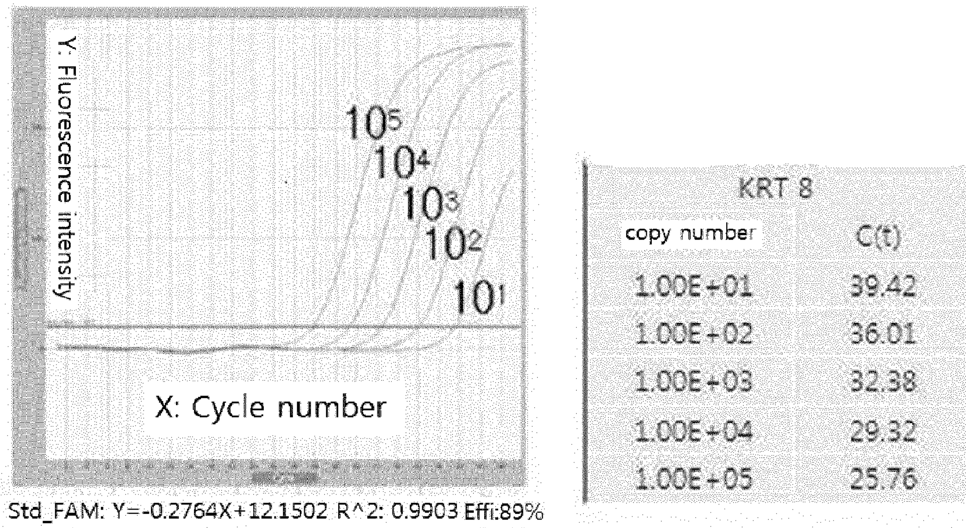
C(t)
0

Fig. 12



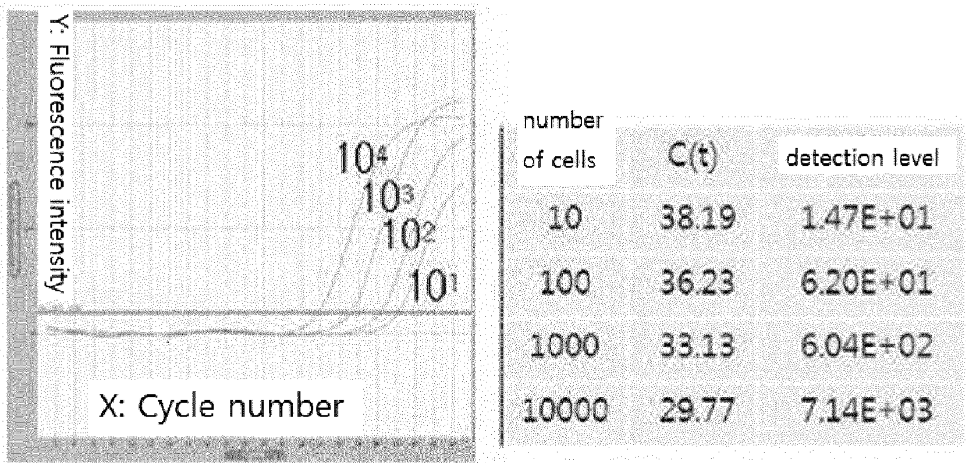
	C(t)
A	32.3/32.2
B	36.5/37.8
C	—
D	—

Fig. 13a



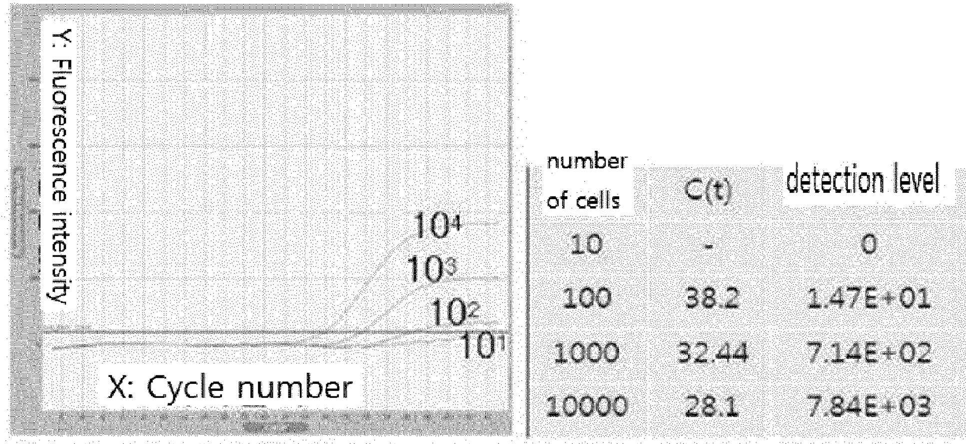
KRT 8	
copy number	C(t)
1.00E+01	39.42
1.00E+02	36.01
1.00E+03	32.38
1.00E+04	29.32
1.00E+05	25.76

Fig. 13b



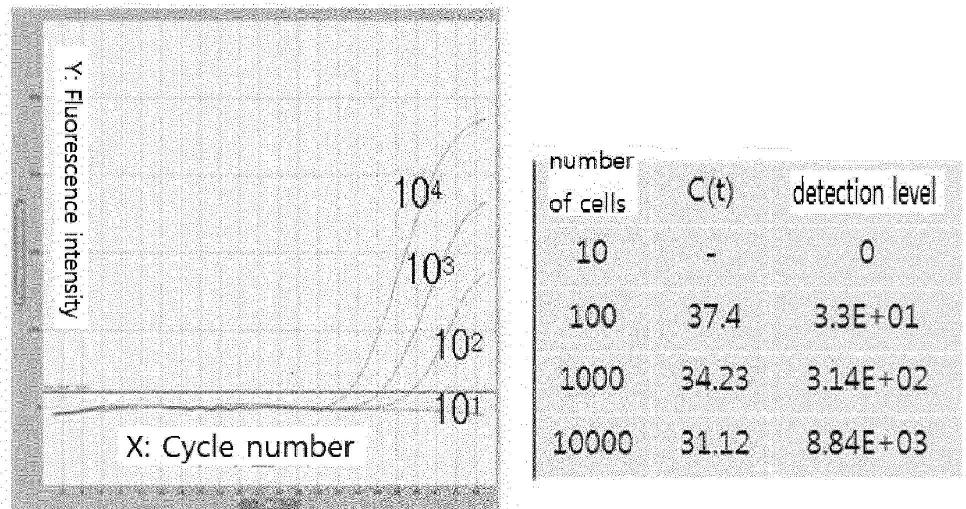
number of cells	C(t)	detection level
10	38.19	1.47E+01
100	36.23	6.20E+01
1000	33.13	6.04E+02
10000	29.77	7.14E+03

Fig. 13c



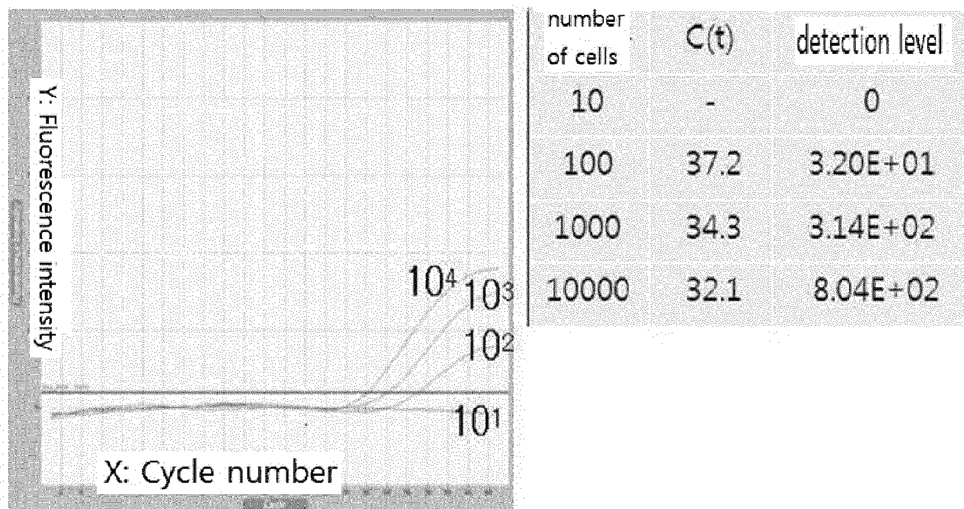
number of cells	C(t)	detection level
10	-	0
100	38.2	1.47E+01
1000	32.44	7.14E+02
10000	28.1	7.84E+03

Fig. 13d

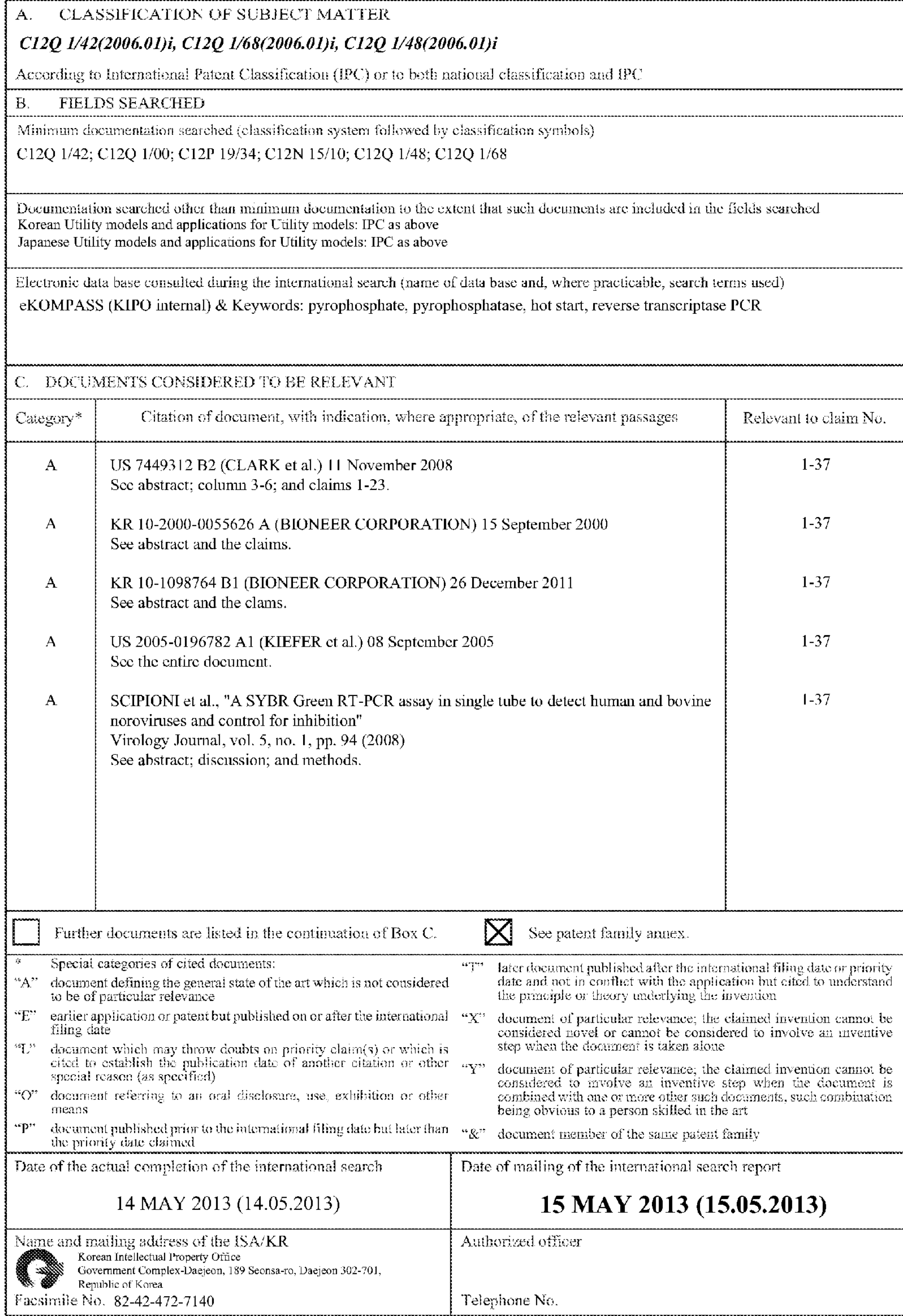


number of cells	C(t)	detection level
10	-	0
100	37.4	3.3E+01
1000	34.23	3.14E+02
10000	31.12	8.84E+03

Fig. 13e



number of cells	C(t)	detection level
10	-	0
100	37.2	3.20E+01
1000	34.3	3.14E+02
10000	32.1	8.04E+02



INTERNATIONAL SEARCH REPORT

International application No. **PCT/KR2013/001935**

A. CLASSIFICATION OF SUBJECT MATTER

C12Q 1/42(2006.01)i, C12Q 1/68(2006.01)i, C12Q 1/48(2006.01)i

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

C12Q 1/42; C12Q 1/00; C12P 19/34; C12N 15/10; C12Q 1/48; C12Q 1/68

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Korean Utility models and applications for Utility models: IPC as above

Japanese Utility models and applications for Utility models: IPC as above

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

eKOMPASS (KIPO internal) & Keywords: pyrophosphate, pyrophosphatase, hot start, reverse transcriptase PCR

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	US 7449312 B2 (CLARK et al.) 11 November 2008 See abstract; column 3-6; and claims 1-23.	1-37
A	KR 10-2000-0055626 A (BIONEER CORPORATION) 15 September 2000 See abstract and the claims.	1-37
A	KR 10-1098764 B1 (BIONEER CORPORATION) 26 December 2011 See abstract and the clams.	1-37
A	US 2005-0196782 A1 (KIEFER et al.) 08 September 2005 See the entire document.	1-37
A	SCIPIONI et al., "A SYBR Green RT-PCR assay in single tube to detect human and bovine noroviruses and control for inhibition" Virology Journal, vol. 5, no. 1, pp. 94 (2008) See abstract; discussion; and methods.	1-37

☐ Further documents are listed in the continuation of Box C. ☒ See patent family annex.

* Special categories of cited documents:

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier application or patent but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art

"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
14 MAY 2013 (14.05.2013)	**15 MAY 2013 (15.05.2013)**

Name and mailing address of the ISA/KR	Authorized officer
Korean Intellectual Property Office Government Complex-Daejeon, 189 Seonsa-ro, Daejeon 302-701, Republic of Korea Facsimile No. 82-42-472-7140	Telephone No.

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INTERNATIONAL SEARCH REPORT
Information on patent family members

International application No.
PCT/KR2013/001935

5

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10 15 20 25 30 35 40 45 50 55

Form PCT/ISA/210 (patent family annex) (July 2009)

REFERENCES CITED IN THE DESCRIPTION

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Electronic Acknowledgement Receipt	
EFS ID:	25812070
Application Number:	14067620
International Application Number:	
Confirmation Number:	4288
Title of Invention:	Nicking and Extension Amplification Reaction for the Exponential Amplification of Nucleic Acids
First Named Inventor/Applicant Name:	Brian K. Maples
Customer Number:	26161
Filer:	Ian J.S. Lodovice/Mary Florczak
Filer Authorized By:	Ian J.S. Lodovice
Attorney Docket Number:	30171-0025002 / ITI-001
Receipt Date:	18-MAY-2016
Filing Date:	30-OCT-2013
Time Stamp:	13:01:11
Application Type:	Utility under 35 USC 111(a)

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2	Foreign Reference	WO2015113828.pdf	3574943 728cd72dbd9a8eb08aaf6dae5c00286bcdffdd23	no	44
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3	Foreign Reference	WO2016004333.pdf	2587786 6de7679f93e6db0682e5f0d7ebe99cb5b3502ed2	no	52
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4	Foreign Reference	WO2003012066.pdf	3509015 a691bad2a10ca76a3aed5b6accdcfadcd191653a	no	64
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5	Foreign Reference	WO2007096182.pdf	1421316 5495f87e37b3b899cd17e3b51f40d207e782ce59	no	37
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6	Foreign Reference	GB2416352A.pdf	1102676 7710a9aa1c325fba11b427aba68cf452428280f4	no	30
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7	Foreign Reference	WO2011085160.pdf	1766141 90399daa943312dea556533643e10e66eb043bbc	no	34
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9	Non Patent Literature	EESR.pdf	381463 83f0fc741db9495e0a6b96d5289fbec337253209	no	10
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10	Non Patent Literature	Arena.pdf	380272 2e8219760a21d4719025001c218fb693bc031ac3	no	10

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11	Non Patent Literature	ExaminerReport.pdf	168041 330460027b691165c7d381a5b85875792b47ce93	no	5
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New Applications Under 35 U.S.C. 111
If a new application is being filed and the application includes the necessary components for a filing date (see 37 CFR 1.53(b)-(d) and MPEP 506), a Filing Receipt (37 CFR 1.54) will be issued in due course and the date shown on this Acknowledgement Receipt will establish the filing date of the application.

National Stage of an International Application under 35 U.S.C. 371
If a timely submission to enter the national stage of an international application is compliant with the conditions of 35 U.S.C. 371 and other applicable requirements a Form PCT/DO/EO/903 indicating acceptance of the application as a national stage submission under 35 U.S.C. 371 will be issued in addition to the Filing Receipt, in due course.

New International Application Filed with the USPTO as a Receiving Office
If a new international application is being filed and the international application includes the necessary components for an international filing date (see PCT Article 11 and MPEP 1810), a Notification of the International Application Number and of the International Filing Date (Form PCT/RO/105) will be issued in due course, subject to prescriptions concerning national security, and the date shown on this Acknowledgement Receipt will establish the international filing date of the application.

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APPLICATION NO.	FILING DATE	FIRST NAMED INVENTOR	ATTORNEY DOCKET NO.	CONFIRMATION NO.
14/067,620	10/30/2013	Brian K. Maples	30171-0025002 / ITI-001	4288

26161 7590 02/11/2016
FISH & RICHARDSON P.C. (BO)
P.O. BOX 1022
MINNEAPOLIS, MN 55440-1022

EXAMINER	
BERTAGNA, ANGELA MARIE	

ART UNIT	PAPER NUMBER
1637	

NOTIFICATION DATE	DELIVERY MODE
02/11/2016	ELECTRONIC

Please find below and/or attached an Office communication concerning this application or proceeding.

The time period for reply, if any, is set in the attached communication.

Notice of the Office communication was sent electronically on above-indicated "Notification Date" to the following e-mail address(es):

PATDOCTC@fr.com

PTOL-90A (Rev. 04/07)

Office Action Summary	**Application No.** 14/067,620	**Applicant(s)** MAPLES ET AL.	
	Examiner Angela M. Bertagna	**Art Unit** 1637	**AIA (First Inventor to File) Status** No

-- The MAILING DATE of this communication appears on the cover sheet with the correspondence address --

Period for Reply

A SHORTENED STATUTORY PERIOD FOR REPLY IS SET TO EXPIRE *3* MONTHS FROM THE MAILING DATE OF THIS COMMUNICATION.

- Extensions of time may be available under the provisions of 37 CFR 1.136(a). In no event, however, may a reply be timely filed after SIX (6) MONTHS from the mailing date of this communication.
- If NO period for reply is specified above, the maximum statutory period will apply and will expire SIX (6) MONTHS from the mailing date of this communication.
- Failure to reply within the set or extended period for reply will, by statute, cause the application to become ABANDONED (35 U.S.C. § 133). Any reply received by the Office later than three months after the mailing date of this communication, even if timely filed, may reduce any earned patent term adjustment. See 37 CFR 1.704(b).

Status

1)☒ Responsive to communication(s) filed on *7/27/15*.

☐ A declaration(s)/affidavit(s) under **37 CFR 1.130(b)** was/were filed on _____.

2a)☐ This action is **FINAL**. 2b)☒ This action is non-final.

3)☐ An election was made by the applicant in response to a restriction requirement set forth during the interview on _____; the restriction requirement and election have been incorporated into this action.

4)☐ Since this application is in condition for allowance except for formal matters, prosecution as to the merits is closed in accordance with the practice under *Ex parte Quayle*, 1935 C.D. 11, 453 O.G. 213.

Disposition of Claims*

5)☒ Claim(s) *67-83,85-88,90-92 and 95-107* is/are pending in the application.

5a) Of the above claim(s) *74,75,78,79,81,82 and 95* is/are withdrawn from consideration.

6)☐ Claim(s) _____ is/are allowed.

7)☒ Claim(s) *67-73,76,77,80,83,85-88,90-92 and 96-107* is/are rejected.

8)☐ Claim(s) _____ is/are objected to.

9)☐ Claim(s) _____ are subject to restriction and/or election requirement.

* If any claims have been determined allowable, you may be eligible to benefit from the **Patent Prosecution Highway** program at a participating intellectual property office for the corresponding application. For more information, please see http://www.uspto.gov/patents/init_events/pph/index.jsp or send an inquiry to PPHfeedback@uspto.gov.

Application Papers

10)☐ The specification is objected to by the Examiner.

11)☐ The drawing(s) filed on _____ is/are: a)☐ accepted or b)☐ objected to by the Examiner.

Applicant may not request that any objection to the drawing(s) be held in abeyance. See 37 CFR 1.85(a).

Replacement drawing sheet(s) including the correction is required if the drawing(s) is objected to. See 37 CFR 1.121(d).

Priority under 35 U.S.C. § 119

12)☐ Acknowledgment is made of a claim for foreign priority under 35 U.S.C. § 119(a)-(d) or (f).

Certified copies:

a)☐ All b)☐ Some** c)☐ None of the:

1.☐ Certified copies of the priority documents have been received.

2.☐ Certified copies of the priority documents have been received in Application No. _____.

3.☐ Copies of the certified copies of the priority documents have been received in this National Stage application from the International Bureau (PCT Rule 17.2(a)).

** See the attached detailed Office action for a list of the certified copies not received.

Attachment(s)

1) ☒ Notice of References Cited (PTO-892)

2) ☒ Information Disclosure Statement(s) (PTO/SB/08a and/or PTO/SB/08b) Paper No(s)/Mail Date _____.

3) ☐ Interview Summary (PTO-413) Paper No(s)/Mail Date. _____ .

4) ☐ Other: _____.

DETAILED ACTION

Notice of Pre-AIA or AIA Status

1. The present application is being examined under the pre-AIA first to invent provisions.

Continued Examination Under 37 CFR 1.114

2. A request for continued examination under 37 CFR 1.114, including the fee set forth in 37 CFR 1.17(e), was filed in this application after final rejection. Since this application is eligible for continued examination under 37 CFR 1.114, and the fee set forth in 37 CFR 1.17(e) has been timely paid, the finality of the previous Office action has been withdrawn pursuant to 37 CFR 1.114. Applicant's submission filed on July 27, 2015 has been entered.

Claims 67-83, 85-88, 90-92, and 95-107 are pending. Claims 67-73, 76, 77, 80, 83, 85-88, 90-92, and 96-107 are under examination. Claims 74, 75, 78, 79, 81, 82, and 95 remain withdrawn as being drawn to a non-elected species.

The following includes new grounds of rejection necessitated by Applicant’s amendments to the claims. Any previously made rejections or objections not reiterated below have been withdrawn.

Response to Arguments

3. Applicant’s arguments filed on July 27, 2015 have been fully considered.

Rejection of claims 67-73, 76, 77, 80, 83, and 85-92 under pre-AIA 35 U.S.C. 112, first paragraph (new matter)

Applicant argues that the rejection should be withdrawn in view of the amendment to claim 67, which replaces "denaturation step" with "thermal denaturation step" (page 10). This argument was persuasive. The rejection has been withdrawn.

Rejection of claims 67-73, 76, 77, 80, 83, and 85-92 under pre-AIA 35 U.S.C. 103(a) as being unpatentable over Wick et al. (US 6,063,604) in view of Kong et al. (US 6,191,267) and further in view of Yao et al. (US 2009/0092967)

The rejection has been withdrawn in view of the amendments to independent claim 67, which replace “denaturation step” with “thermal denaturation step”. In view of this amendment, claims 67-73, 76, 77, 80, 83, 85-88, 90-92, and 96-98 are rejected under pre-AIA 35 U.S.C. 103(a) as being unpatentable over Wick in view of Kong. Also, claims 99-107 are rejected under pre-AIA 35 U.S.C. 103(a) as being unpatentable over Wick in view of Kong and further in view of Yao et al. (US 2008/0096257 A1).

Applicant’s arguments on pages 11-13 have been fully considered since they apply to the new grounds of rejection set forth below.

Applicant first argues that the ordinary artisan would not have had a motivation or a reasonable expectation of success in combining Wick, Kong, and Yao (pages 11-12). Applicant argues that Yao's teachings concerning eliminating an initial thermal denaturation step are inapplicable to Wick because the teachings of Yao relate to preparing a single-stranded target nucleic acid to be subjected to SDA from a double-stranded nucleic acid, whereas the nucleic acid to be amplified by SDA in Wick is already single-stranded (pages 11-12). Therefore, Applicant argues, the ordinary artisan would have had neither a motivation nor a reasonable expectation of success in combining the references (pages 11-12).

These arguments have been fully considered, but they were not persuasive. It is first noted that the amended claims, with the exception of new claim 107, only prohibit an initial thermal denaturation step. Wick is not limited to an initial thermal denaturation step and teaches that an initial pH denaturation may be used (column 25, lines 28-33). Second, the teachings of Yao concerning eliminating an initial thermal denaturation step are, in fact, quite relevant to the method of Wick. Wick is indeed cited for its disclosure of viral RNA from a single-stranded RNA virus as the target nucleic acid for SDA, but viral RNA from single-stranded RNA viruses was known to possess double-stranded portions in the form of secondary structure (see, for example, Brown et al. (Nucleic Acids Research (1992) 20: 5041-5045) at Figure 3 and page 5044; see also Baudin et al. (The EMBO Journal (1994) 13: 3158-3165) at pages 3159-3162 and Figure 8). Therefore, the ordinary artisan practicing the method of Wick would have been motivated to include a pre-amplification denaturation step to eliminate any secondary structure present in the viral RNA to be amplified, and the teachings of Yao concerning elimination of this step to result in a simpler method with fewer steps would have been highly relevant to the ordinary artisan practicing the method of Wick. Thus, there is a clear motivation to eliminate a pre-amplification denaturation step in view of the teachings of the references in combination with the knowledge generally available to the ordinary artisan. Lastly, in response to Applicant's argument concerning reasonable expectation of success, it is not at all clear as to why the ordinary artisan would not have expected anything *other* than the result of successful amplification when eliminating an initial denaturation step as suggested by Yao. Wick and Yao are each directed to SDA, and there is nothing in the disclosure of Yao to suggest that an initial

denaturation step can only be eliminated when single-stranded DNA prepared from double-stranded DNA is subjected to SDA.

Applicant also argues that the claimed invention is associated with unexpected results and points to Exhibits 5 and 6, which describe the ability of a commercial product based on the claimed invention to provide high yields of amplified product and sensitive detection in 15 minutes or less (page 12).

Applicant's arguments concerning unexpected results have been fully considered, but they were not persuasive. First, the result of high yield and sensitive detection in as little as 15 minutes does not appear to be unexpected in view of the references cited in the new rejection set forth below, particularly since the newly cited Yao reference teaches that SDA can be performed in about 10 minutes (paragraphs 31 and 48). Second, the results described in Exhibits 5 and 6 are is not commensurate in scope with the claimed invention at least for the following reasons. First, Exhibits 5 and 6 only state that the commercial product could generate a detectable amplification product in 15 minutes or less. It is not at all clear that same result would be obtained with the shorter time ranges recited in claims 99-107. Also, not all of the claims require a particular amplification time. Only claims 99-107 require amplification for a particular length of time. It is also not clear that the results described in Exhibits 5 and 6 would extend to nucleic acids other than Influenza nucleic acids. Further, since Exhibits 5 and 6 do not describe the conditions of the commercialized NEAR assay, it also cannot be determined whether particular reaction conditions are required to obtain the result.

Double Patenting

Applicant requests that the issue be held in abeyance until allowable subject matter has been identified (page 13). Applicant's remarks concerning the rejections are acknowledged. They have been maintained with modifications necessitated by the amendment since they are still applicable.

Claim Rejections - 35 USC § 112 (New Matter)

4. The following is a quotation of the first paragraph of 35 U.S.C. 112(a):

> (a) IN GENERAL.—The specification shall contain a written description of the invention, and of the manner and process of making and using it, in such full, clear, concise, and exact terms as to enable any person skilled in the art to which it pertains, or with which it is most nearly connected, to make and use the same, and shall set forth the best mode contemplated by the inventor or joint inventor of carrying out the invention.

The following is a quotation of the first paragraph of pre-AIA 35 U.S.C. 112:

> The specification shall contain a written description of the invention, and of the manner and process of making and using it, in such full, clear, concise, and exact terms as to enable any person skilled in the art to which it pertains, or with which it is most nearly connected, to make and use the same, and shall set forth the best mode contemplated by the inventor of carrying out his invention.

Claim 107 is rejected under 35 U.S.C. 112(a) or 35 U.S.C. 112 (pre-AIA), first paragraph, as failing to comply with the written description requirement. The claim contains subject matter which was not described in the specification in such a way as to reasonably convey to one skilled in the relevant art that the inventor or a joint inventor, or for pre-AIA the inventor(s), at the time the application was filed, had possession of the claimed invention. **This is a new matter rejection.**

New claim 107 is drawn to a method for amplifying a target nucleic acid sequence. The claim recites preparing a mixture comprising the target nucleic acid, a polymerase, a nicking enzyme, a first oligonucleotide comprising a nicking site and nicking enzyme binding site, and a second oligonucleotide comprising a nicking site and a nicking enzyme binding site and

subjecting the mixture to essentially isothermal conditions to amplify the target nucleic acid. The claim requires the mixture to be prepared "without first subjecting the target nucleic acid to a denaturation step associated with amplification of the target polynucleotide sequence".

Applicant states that the limitation finds support in original claim 67 and Examples 10-11 (page 9).

The original disclosure, including the portions cited by Applicant, has been reviewed, but support was not found for omitting a generic denaturation step as recited in claim 107. The original disclosure only provides support for omitting a heat denaturation step (see page 4, lines 16-29, for example). Accordingly, claim 107 contains new matter.

Claim Rejections - 35 USC § 103

5. The following is a quotation of pre-AIA 35 U.S.C. 103(a) which forms the basis for all obviousness rejections set forth in this Office action:

> (a) A patent may not be obtained though the invention is not identically disclosed or described as set forth in section 102 of this title, if the differences between the subject matter sought to be patented and the prior art are such that the subject matter as a whole would have been obvious at the time the invention was made to a person having ordinary skill in the art to which said subject matter pertains. Patentability shall not be negatived by the manner in which the invention was made.

This application currently names joint inventors. In considering patentability of the claims under pre-AIA 35 U.S.C. 103(a), the examiner presumes that the subject matter of the various claims was commonly owned at the time any inventions covered therein were made absent any evidence to the contrary. Applicant is advised of the obligation under 37 CFR 1.56 to point out the inventor and invention dates of each claim that was not commonly owned at the time a later invention was made in order for the examiner to consider the applicability of pre-

AIA 35 U.S.C. 103(c) and potential pre-AIA 35 U.S.C. 102(e), (f) or (g) prior art under pre-AIA 35 U.S.C. 103(a).

6. Claims 67-73, 76, 77, 80, 83, 85-88, 90-92, and 96-98 are rejected under pre-AIA 35 U.S.C. 103(a) as being unpatentable over Wick et al. (US 6,063,604) in view of Kong et al. (US 6,191,267).

Regarding claims 67-73, 76, 77, 80, and 83, Wick teaches a method that comprises subjecting a mixture of the following components to an amplification reaction: (i) a target nucleic acid, (ii) a polymerase, (iii) a restriction endonuclease capable of nicking at a hemi-modified recognition site, and (iv) two oligonucleotides, each comprising a recognition sequence for the restriction enzyme of (iii) (see, for example, Figure 1, column 10, line 39 – column 11, line 67, column 13, lines 7-52, and columns 25-28 ("Segregation and/or Amplification Scheme"); see also the Examples at columns 28-31). In the amplification method of Wick, the steps set forth in parts (a)-(f) of claim 68, parts (i) and (ii) of claim 69, and parts (a)-(c) of claim 70 occur (see Figures 1a-1b and the accompanying discussion at columns 25-28).The reference teaches that the target nucleic acid may be single-stranded viral RNA from an animal pathogen (see, for example, column 12, lines 17-21, 24-30, 34-38, and 62-66) and that samples containing the target nucleic acid may be isolated from tissue (e.g., liver, lung, kidney, and spleen), blood, tears, feces, urine, sputum, mucus, bone marrow, tissues or saliva samples obtained from humans suspected of having been in contact with the virus (see, for example, column 12, lines 24-30 and 34-38 as well as column 13, lines 53-63). Wick also teaches that the method may be conducted without first subjecting the target nucleic acid to a thermal denaturation step (column 25, lines 28-33, where an initial pH denaturation step is taught).

Regarding claim 86, Wick teaches that the nicking step does not result in nicking within the target polynucleotide sequence (see Figure 1b, for example).

Regarding claims 87 and 88, Wick teaches isothermal amplification at a temperature within the claimed range (see, for example, column 13, lines 7-52, column 24, lines 33-67, and column 29, lines 2-8).

Regarding claims 91 and 92, Wick teaches detecting the amplification product using at least one of the required methods (see, for example, column 29, lines 11-44).

In the method of Wick, modified nucleotides (e.g., phosphorothioated nucleotides) are used to create a hemi-modified nucleic acid substrate for nicking (column 15, lines 17-53). As a result, the reference fails to teach the use of a nicking enzyme (e.g., Nt.BstNBI) as required by all of the claims. Wick also does not teach that the amplification reaction is performed at a temperature that is higher than the melting temperature of the duplex formed between the first oligonucleotide and the target polynucleotide as required by claim 90. Wick also does not disclose the yields recited in claims 96-98.

However, Kong describes methods for making and using Nt.BstNBI, which is a nicking enzyme (abstract and column 4, lines 21-50; see also Example 4 at column 14). The reference further teaches that the modified nucleotides typically used in strand displacement amplification are expensive and poorly incorporated by DNA polymerase, but that the use of a nicking enzyme eliminates the need for their use (column 2, lines 4-45).

It would have been *prima facie* obvious for one of ordinary skill in the art at the time of the invention to conduct the isothermal amplification method of Wick using the Nt.BstNBI nicking enzyme described by Kong. The ordinary artisan would have been motivated to do so

since Kong taught that using a nicking enzyme eliminates the need to use more expensive and poorly incorporated modified nucleotides in isothermal amplification reactions. The ordinary artisan would have had a reasonable expectation of success since Kong described methods for obtaining the enzyme (Examples 1-3 at columns 6-14) as well as a method for using it in an isothermal strand displacement amplification reaction (Example 4 at column 14).

Further regarding claim 90, it also would have been *prima facie* obvious for one of ordinary skill in the art at the time of the invention to conduct the amplification step in the method suggested by the teachings of Wick and Kong at a temperature that is higher than the melting temperature of the duplex formed between the first oligonucleotide and the target polynucleotide. The ordinary artisan would have recognized that the temperature at which the amplification reaction is conducted is a results-effective variable and, accordingly, would have been motivated to conduct routine experimentation to determine an optimal temperature with a reasonable expectation of success. As discussed in MPEP 2144.05, performing routine experimentation to optimize results-effective variables is *prima facie* obvious in the absence of secondary considerations. In this case, no evidence of unexpected results has been presented.

Finally, regarding new claims 96-98, the references do not report the yield of the disclosed amplification reactions. However, since the method suggested by the references contains all of the required steps, the ability to obtain the claimed yields is presumed to be an inherent property. It is also generally desirable and routine to optimize or modify a method such that it is capable of producing a desired amount of product.

Thus, the methods of claims 67-73, 76, 77, 80, 83, 85-88, 90-92, and 96-98 are *prima facie* obvious over Wick in view of Kong.

7. Claims 99-107 are rejected under pre-AIA 35 U.S.C. 103(a) as being unpatentable over Wick et al. (US 6,063,604) in view of Kong et al. (US 6,191,267) and further in view of Yao et al. (US 2008/0096257 A1).

As discussed above, the teachings of Wick and Kong render obvious the methods of claims 67-73, 76, 77, 80, 83, 85-88, 90-92, and 96-98.

Regarding claims 99-106, neither Wick nor Kong teaches an amplification time within the claimed ranges.

The teachings of Wick in view of Kong also render obvious the method of claim 107 except for the claimed amplification time and the elimination of any denaturation step associated with the amplification reaction for the reasons set forth above.

However, Yao describes a one-step, real-time SDA method in which the amplification time is ten minutes (paragraphs 31 and 48). The teachings of Yao also indicate that amplification products may be detected after approximately seven minutes (Figure 6 and paragraph 48).

It would have been *prima facie* obvious for the ordinary artisan to modify the SDA reaction suggested by Wick and Kong to be a one-step, real-time reaction. The ordinary artisan would have been motivated to do so since Yao taught that such a method could be completed in as few as ten minutes (paragraphs 31 and 48). In the absence of unexpected results, the teachings of Yao also render obvious the amplification times recited in claims 102-104 for the following reasons. First, the results in Figure 6 of Yao indicate that amplified product may be detected in as little as seven minutes. Second, the teachings of Yao provide motivation for the ordinary artisan to conduct routine experimentation to further reduce the amplification time in order to further increase the efficiency of the assay. The ordinary artisan would have had a reasonable

expectation of success since factors influencing the efficiency of amplification reactions were well known at the time of the invention. Thus, the methods of claims 99-107 are *prima facie* obvious over Wick in view of Kong and further in view of Yao.

Double Patenting

8. The nonstatutory double patenting rejection is based on a judicially created doctrine grounded in public policy (a policy reflected in the statute) so as to prevent the unjustified or improper timewise extension of the “right to exclude” granted by a patent and to prevent possible harassment by multiple assignees. A nonstatutory double patenting rejection is appropriate where the claims at issue are not identical, but at least one examined application claim is not patentably distinct from the reference claim(s) because the examined application claim is either anticipated by, or would have been obvious over, the reference claim(s). See, e.g., *In re Berg*, 140 F.3d 1428, 46 USPQ2d 1226 (Fed. Cir. 1998); *In re Goodman*, 11 F.3d 1046, 29 USPQ2d 2010 (Fed. Cir. 1993); *In re Longi*, 759 F.2d 887, 225 USPQ 645 (Fed. Cir. 1985); *In re Van Ornum*, 686 F.2d 937, 214 USPQ 761 (CCPA 1982); *In re Vogel*, 422 F.2d 438, 164 USPQ 619 (CCPA 1970); and *In re Thorington*, 418 F.2d 528, 163 USPQ 644 (CCPA 1969).

A timely filed terminal disclaimer in compliance with 37 CFR 1.321(c) or 1.321(d) may be used to overcome an actual or provisional rejection based on a nonstatutory double patenting ground provided the reference application or patent either is shown to be commonly owned with this application, or claims an invention made as a result of activities undertaken within the scope of a joint research agreement. A terminal disclaimer must be signed in compliance with 37 CFR 1.321(b).

The USPTO internet Web site contains terminal disclaimer forms which may be used. Please visit http://www.uspto.gov/forms/. The filing date of the application will determine what form should be used. A web-based eTerminal Disclaimer may be filled out completely online using web-screens. An eTerminal Disclaimer that meets all requirements is auto-processed and approved immediately upon submission. For more information about eTerminal Disclaimers, refer to http://www.uspto.gov/patents/process/file/efs/guidance/eTD-info-I.jsp.

9. Claims 67-73, 76, 77, 80, 83, 85-88, 90-92, and 96-106 are provisionally rejected on the ground of nonstatutory double patenting as being unpatentable over claims 67-69, 74-76, 79, 80, 83, 85, 87-90, 92-94, and 97-106 of copending Application No. 14/067,623. Although the claims at issue are not identical, they are not patentably distinct from each other because the claims of the '623 application overlap in scope with the claimed methods and recite all of their limitations.

This is a provisional nonstatutory double patenting rejection because the patentably indistinct claims have not in fact been patented.

10. Claim 107 is provisionally rejected on the ground of nonstatutory double patenting as being unpatentable over claims 67-69, 74-76, 79, 80, 83, 85, 87-90, 92-94, and 97-106 of copending Application No. 14/067,623 in view of Yao et al. (US 2008/0096257 A1).

The claims of the ‘623 application teach all of the elements of the instant claim 107 (see, for example, claims 67 and 97 of the '623 application) except for the requirement for the method to lack a denaturation step associated with the amplification reaction.

However, Yao describes a one-step, real-time SDA method in which the amplification time is ten minutes (paragraphs 31 and 48). This method does not include a denaturation step associated with the amplification reaction.

It would have been *prima facie* obvious for the ordinary artisan practicing the method recited in the claims of the ‘623 application to omit a denaturation step associated with the amplification reaction. The ordinary artisan would have been motivated to do so in view of the teachings of Yao cited above, which indicate that denaturation may be eliminated to achieve a faster, simpler, and more efficient amplification method. Thus, the instant claim 107 is not patentably distinct from the claims of the '623 application in view of Yao.

This is a provisional nonstatutory double patenting rejection because the patentably indistinct claims have not in fact been patented.

11. Claims 67-73, 76, 77, 80, 83, 85-88, 90-92, and 96-106 are provisionally rejected on the ground of nonstatutory double patenting as being unpatentable over claims 1-9, 12, 14-17, 19-41, and 44-46 of copending Application No. 12/173,020.

Although the claims at issue are not identical, they are not patentably distinct from each other because the claims of the ‘020 application are also drawn to an isothermal amplification method that omits an initial thermal denaturation step and comprises the use of nicking enzymes and primers having nicking enzyme recognition and cleavage sites (see, in particular, claims 1, 41, 42, 44, and 47 of the ‘020 application). The claims of the '020 application also suggest the use of single-stranded viral RNA in a sample from an animal as the target polynucleotide (see, in particular, claims 9, 12, 14, 45, and 46 of the ‘020 application) as well as amplification time

ranges and yields that overlap in scope with or are very close to the claimed amplification time ranges and yields (see, in particular, claims 1, 26, 27, 35-39, and 44 of the ‘020 application). Further regarding the instant claim 77, mucus, saliva, and sputum were generally known in the art to be useful samples for nucleic acid amplification procedures. Accordingly, this claim is also not patentably distinct from the claims of the '020 application. Further regarding the instant claim 90, although the claims of the '020 application do not state that the amplification step is performed at a temperature that is higher than the melting temperature of the duplex formed between the first oligonucleotide template and the target polynucleotide, the ordinary artisan would have recognized that the temperature at which the amplification reaction is conducted is a results-effective variable and, accordingly, would have been motivated to conduct routine experimentation to determine an optimal temperature with a reasonable expectation of success. As discussed in MPEP 2144.05, performing routine experimentation to optimize results-effective variables is *prima facie* obvious in the absence of secondary considerations. In this case, no evidence of unexpected results has been presented. Thus, the instant claims 67-73, 76, 77, 80, 83, 85-88, 90-92, and 96-106 are not patentably distinct from claims 1-9, 12, 14-17, 19-41, and 44-46 of the ‘020 application.

This is a provisional nonstatutory double patenting rejection because the patentably indistinct claims have not in fact been patented.

12. Claim 107 is provisionally rejected on the ground of nonstatutory double patenting as being unpatentable over claims 1-9, 12, 14-17, 19-41, and 44-46 of copending Application No. 12/173,020 in view of Yao et al. (US 2008/0096257 A1).

The claims of the ‘020 application teach all of the elements of the instant claim 107 (see, for example, claims 1, 9, 12, 14, and 38 of the ‘020 application) except for the requirement for the method to lack a denaturation step associated with the amplification reaction.

However, Yao describes a one-step, real-time SDA method in which the amplification time is ten minutes (paragraphs 31 and 48). This method does not include a denaturation step associated with the amplification reaction.

It would have been *prima facie* obvious for the ordinary artisan practicing the method recited in the claims of the ‘020 application to omit a denaturation step associated with the amplification reaction. The ordinary artisan would have been motivated to do so in view of the teachings of Yao cited above, which indicate that denaturation may be eliminated to achieve a faster, simpler, and more efficient amplification method. Thus, the instant claim 107 is not patentably distinct from the claims of the '020 application in view of Yao.

This is a provisional nonstatutory double patenting rejection because the patentably indistinct claims have not in fact been patented.

13. Claims 67-73, 76, 77, 80, 83, 86-88, 90-92, and 96-98 are provisionally rejected on the ground of nonstatutory double patenting as being unpatentable over claims 125-130 of copending Application No. 11/778,018 in view of Wick et al. (US 6,063,604).

The instant claims are drawn to a method for isothermal amplification of a single-stranded viral RNA target that comprises the use of nicking enzymes. The claimed method also does not contain a pre-amplification thermal denaturation step. Claims 125-130 of the ‘018 application are also drawn to a method for isothermal amplification of a single-stranded RNA

target that comprises the use of nicking enzymes and lacks a pre-amplification thermal denaturation step. The claims of the ‘018 application disclose or suggest all of the limitations of the instant claims 67-70, 80, and 86-88, but they do not state that the RNA target is a single-stranded viral RNA contained in a sample obtained from an animal as required by the instant claims. The claims of the '018 application also do not state that the method further comprises detection of the amplification product as required by the instant claims 91 and 92. Further, the claims of the ‘018 application do not teach the requirements of the instant claim 90 or yields within the ranges recited in the instant claims 96-98.

Wick teaches a method that comprises subjecting a mixture of the following components to an iosothermal amplification reaction: (i) a target nucleic acid, (ii) a polymerase, (iii) a restriction endonuclease capable of nicking at a hemi-modified recognition site, and (iv) two oligonucleotides, each comprising a recognition sequence for the restriction enzyme of (iii) (see, for example, Figure 1, column 10, line 39 – column 11, line 67, column 13, lines 7-52, and columns 25-28 (“Segregation and/or Amplification Scheme”); see also the Examples at columns 28-31). The reference teaches that the target nucleic acid may be single-stranded viral RNA from an animal pathogen (see, for example, column 12, lines 17-21, 24-30, 34-38, and 62-66) and that samples containing the target nucleic acid may be isolated from tissue (e.g., liver, lung, kidney, and spleen), blood, tears, feces, urine, sputum, mucus, bone marrow, tissues or saliva samples obtained from humans suspected of having been in contact with the virus (see, for example, column 12, lines 24-30 and 34-38 as well as column 13, lines 53-63).

Regarding claims 91 and 92, Wick teaches detecting the amplification product using at least one of the required methods (see, for example, column 29, lines 11-44).

It would have been *prima facie* obvious for one of ordinary skill in the art at the time of the invention to conduct the isothermal amplification method recited in the claims of the ‘018 application using a single-stranded viral RNA contained in a sample from an animal as the target nucleic acid. The ordinary artisan would have been motivated to do so with a reasonable expectation of success in view of the teachings of Wick, which indicate that such samples and targets may be used in a similar isothermal amplification method comprising nicking and strand displacement. The ordinary artisan also would have been motivated to detect the amplification product using a method described by Wick since detection of amplification products was routine and conventional in the art at the time of the invention. Further regarding the instant claim 90, it also would have been *prima facie* obvious for one of ordinary skill in the art at the time of the invention to conduct the amplification step at a temperature that is higher than the melting temperature of the duplex formed between the first oligonucleotide and the target polynucleotide. The ordinary artisan would have recognized that the temperature at which the amplification reaction is conducted is a results-effective variable and, accordingly, would have been motivated to conduct routine experimentation to determine an optimal temperature with a reasonable expectation of success. As discussed in MPEP 2144.05, performing routine experimentation to optimize results-effective variables is *prima facie* obvious in the absence of secondary considerations. In this case, no evidence of unexpected results has been presented. Finally, further regarding the instant claims 96-98, the claims of the ‘018 application and Wick do not report the yield of the amplification reactions. However, since the method suggested by the references contains all of the required steps, the ability to obtain the claimed yields is presumed to be an inherent property. It is also generally desirable and routine to optimize or modify a

method such that it is capable of producing a desired amount of product. Thus, the instant claims 67-73, 76, 77, 80, 83, 86-88, 90-92, and 96-98 are not patentably distinct from claims 125-130 of the '018 application in view of Wick and further in view of Yao.

This is a provisional nonstatutory double patenting rejection because the patentably indistinct claims have not in fact been patented.

14. Claims 99-107 are provisionally rejected on the ground of nonstatutory double patenting as being unpatentable over claims 125-130 of copending Application No. 11/778,018 in view of Wick et al. (US 6,063,604) and further in view of Yao et al. (US 2008/0096257 A1).

As discussed above, the instant claims 67-73, 76, 77, 80, 83, 86-88, 90-92, and 96-98 are not patentably distinct from claims 125-130 of the '018 application in view of Wick and further in view of Yao.

Regarding the instant claims 99-106, neither the claims of the '018 application nor Wick teaches an amplification time within the claimed ranges.

The claims of the '018 application and Wick also render obvious the instant claim 107 except for the claimed amplification time and the elimination of any denaturation step associated with the amplification reaction for the reasons set forth above.

Yao describes a one-step, real-time SDA method in which the amplification time is ten minutes (paragraphs 31 and 48). The teachings of Yao also indicate that amplification products may be detected after approximately seven minutes (Figure 6 and paragraph 48).

It would have been *prima facie* obvious for the ordinary artisan practicing the method suggested by the claims of the '018 application and Wick to conduct the amplification reaction

for a time within the claimed range and also to omit an amplification-associated denaturation step. The ordinary artisan would have been motivated to do so since Yao taught that an amplification-associated denaturation reaction could be omitted in a similar amplification reaction to obtain a faster, simpler, and more efficient method capable of being completed in as few as ten minutes (paragraphs 31 and 48). Thus, the instant claims 99-107 are not patentably distinct from claims 125-130 of the '018 application in view of Wick and further in view of Yao.

This is a provisional nonstatutory double patenting rejection because the patentably indistinct claims have not in fact been patented.

15. Claim 85 is provisionally rejected on the ground of nonstatutory double patenting as being unpatentable over claims 125-130 of copending Application No. 11/778,018 in view of Wick et al. (US 6,063,604) and further in view of Kong et al. (US 6,191,267).

As discussed above, the instant claims 67-73, 76, 77, 80, 83, 86-88, 90-92, and 96-98 are not patentably distinct from claims 125-130 of the ‘018 application in view of Wick.

Neither Wick nor the claims of the ‘018 application teach the use of Nt.BstNBI as the nicking enzyme as required by the instant claim 85.

Kong describes methods for making and using Nt.BstNBI, which is a nicking enzyme suitable for use in isothermal strand displacement amplification methods comprising a nicking step (abstract and column 4, lines 21-50; see also Example 4 at column 14).

It would have been *prima facie* obvious for the ordinary artisan practicing the method suggested by the claims of the '018 application and Wick to select Nt.BstNBI as the nicking enzyme. The ordinary artisan would have been motivated to do so with a reasonable expectation

of success since Kong taught that this nicking enzyme was suitable for use in isothermal strand displacement amplification reactions comprising a nicking step and also eliminated the need to use expensive and poorly incorporated modified nucleotides. Thus, the instant claim 87 is not patentably distinct from claims 125-130 of the ‘018 application in view of Wick and further in view of Kong.

This is a provisional nonstatutory double patenting rejection because the patentably indistinct claims have not in fact been patented.

Conclusion

16. No claims are currently allowable.

Any inquiry concerning this communication or earlier communications from the examiner should be directed to Angela M. Bertagna whose telephone number is (571)272-8291. The examiner can normally be reached on Monday-Friday, 9-5.

If attempts to reach the examiner by telephone are unsuccessful, the examiner’s supervisor, Gary Benzion can be reached on (571)272-0782. The fax phone number for the organization where this application or proceeding is assigned is 571-273-8300.

Information regarding the status of an application may be obtained from the Patent Application Information Retrieval (PAIR) system. Status information for published applications may be obtained from either Private PAIR or Public PAIR. Status information for unpublished applications is available through Private PAIR only. For more information about the PAIR system, see http://pair-direct.uspto.gov. Should you have questions on access to the Private PAIR system, contact the Electronic Business Center (EBC) at 866-217-9197 (toll-free). If you would like assistance from a USPTO Customer Service Representative or access to the automated information system, call 800-786-9199 (IN USA OR CANADA) or 571-272-1000.

/Angela M. Bertagna/
Primary Examiner, Art Unit 1637

Notice of References Cited	Application/Control No. 14/067,620	Applicant(s)/Patent Under Reexamination MAPLES ET AL.	
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U.S. PATENT DOCUMENTS

*		Document Number Country Code-Number-Kind Code	Date MM-YYYY	Name	CPC Classification	US Classification
	A	US-				
	B	US-				
	C	US-				
	D	US-				
	E	US-				
	F	US-				
	G	US-				
	H	US-				
	I	US-				
	J	US-				
	K	US-				
	L	US-				
	M	US-				

FOREIGN PATENT DOCUMENTS

*		Document Number Country Code-Number-Kind Code	Date MM-YYYY	Country	Name	CPC Classification
	N					
	O					
	P					
	Q					
	R					
	S					
	T					

NON-PATENT DOCUMENTS

*		Include as applicable: Author, Title Date, Publisher, Edition or Volume, Pertinent Pages)
	U	Brown et al. Nucleic Acids Research (1992) 20: 5041-5045.
	V	Baudin et al. The EMBO Journal (1994) 13: 3158-3165.
	W	
	X	

*A copy of this reference is not being furnished with this Office action. (See MPEP § 707.05(a).)
Dates in MM-YYYY format are publication dates. Classifications may be US or foreign.

EAST Search History

EAST Search History (Prior Art)

Ref #	Hits	Search Query	DBs	Default Operator	Plurals	Time Stamp
L4	289	((strand adj1 displac$) or sda) same ("15" adj1 (min or minutes))	US-PGPUB; USPAT; FPRS; EPO; JPO; DERWENT	OR	OFF	2016/02/04 16:41
L5	38	((strand adj1 displac$) or sda) near8 ("15" adj1 (min or minutes))	US-PGPUB; USPAT; FPRS; EPO; JPO; DERWENT	OR	OFF	2016/02/04 16:41
L6	41	((strand adj1 displac$) or sda) near8 ("10" adj1 (min or minutes))	US-PGPUB; USPAT; FPRS; EPO; JPO; DERWENT	OR	OFF	2016/02/04 16:41

EAST Search History (Interference)

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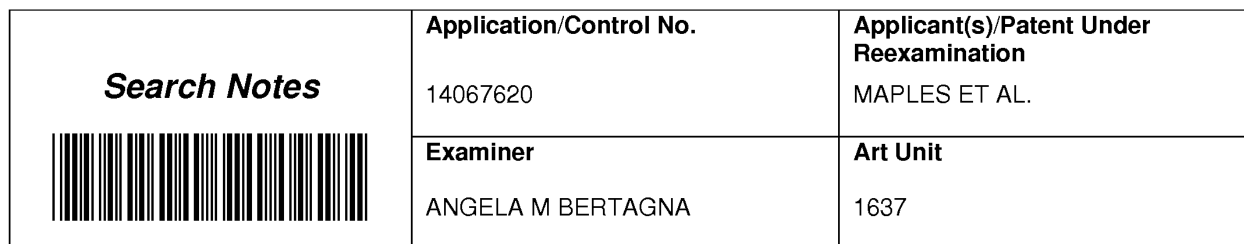
2/4/2016 4:42:51 PM
C:\Users\abertagna\Documents\EAST\Workspaces\14067620\14067620.wsp

Search Notes 	Application/Control No. 14067620	Applicant(s)/Patent Under Reexamination MAPLES ET AL.
	Examiner ANGELA M BERTAGNA	Art Unit 1637

CPC- SEARCHED		
Symbol	Date	Examiner

CPC COMBINATION SETS - SEARCHED		
Symbol	Date	Examiner

US CLASSIFICATION SEARCHED			
Class	Subclass	Date	Examiner

SEARCH NOTES		
Search Notes	Date	Examiner
searched all inventors by name	10/10/2014	amb
EAST search history attached	10/10/2014; 10/14/2014	amb
Google Scholar (search terms included "nicking", "isothermal", and "RNA")	10/10/2014; 10/14/2014	amb
reviewed related cases - 14067623 & 11778018	10/10/2014	amb
updated search	2/18/15; 2/19/15	amb
updated search	2/4/2016	amb

INTERFERENCE SEARCH			
US Class/ CPC Symbol	US Subclass / CPC Group	Date	Examiner

Substitute Disclosure Form / U.S. Department of Commerce Patent and Trademark Office	Attorney Docket No. 30171-0025002	Application No. 14/067,620
Information Disclosure Statement by Applicant (Use several sheets if necessary)	Applicant: Ionian Technologies Inc.	
(37 CFR §1.98(b))	Filing Date: October 30, 2013	Group Art Unit: 1637

U.S. Patent Documents

Examiner Initial	Desig. ID	Document Number	Publication Date	Patentee	Class	Subclass	Filing Date If Appropriate
	1.						

Foreign Patent Documents or Published Foreign Patent Applications

Examiner Initial	Desig. ID	Document Number	Publication Date	Country or Patent Office	Class	Subclass	Translation	
							Yes	No
	2.							

Other Documents (include Author, Title, Date, and Place of Publication)

Examiner Initial	Desig. ID	Document
/A.B./	3.	Summons to Oral Proceedings in corresponding European Application No. 08781827.4, dated January 14, 2016, pages 1-17

Examiner Signature /Angela Bertagna/	Date Considered 02/05/2016
EXAMINER: Initials citation considered. Draw line through citation if not in conformance and not considered. Include copy of this form with next communication to applicant.	

Substitute Disclosure Form

Substitute Disclosure Form U.S. Department of Commerce Patent and Trademark Office	Attorney Docket No. 30171-0025002	Application No. 14/067,620
Information Disclosure Statement by Applicant (Use several sheets if necessary)	Applicant Ionian Technologies Inc.	
(37 CFR §1.98(b))	Filing Date October 30, 2013	Group Art Unit 1637

U.S. Patent Documents							
Examiner Initial	Desig. ID	Document Number	Publication Date	Patentee	Class	Subclass	Filing Date If Appropriate
	1.						

Foreign Patent Documents or Published Foreign Patent Applications								
Examiner Initial	Desig. ID	Document Number	Publication Date	Country or Patent Office	Class	Subclass	Translation Yes	Translation No
/A.B./	2.	WO2007096702	08/30/2007	WIPO				

Other Documents (include Author, Title, Date, and Place of Publication)		
Examiner Initial	Desig. ID	Document
	3.	

Examiner Signature /Angela Bertagna/	Date Considered 02/05/2016
EXAMINER: Initials citation considered. Draw line through citation if not in conformance and not considered. Include copy of this form with next communication to applicant.	

Substitute Disclosure Form

Receipt date: 11/25/2015

14067620 - GAU: 1637

Substitute Disclosure Form / U.S. Department of Commerce Patent and Trademark Office	Attorney Docket No. 30171-0025002	Application No. 14/067,620
Information Disclosure Statement by Applicant (Use several sheets if necessary)	Applicant Ionian Technologies Inc.	
(37 CFR §1.98(b))	Filing Date October 30, 2013	Group Art Unit 1637

U.S. Patent Documents

Examiner Initial	Desig. ID	Document Number	Publication Date	Patentee	Class	Subclass	Filing Date If Appropriate
/A.B./	1.	2008/0096257	04/24/2008	Yao et al.			

Foreign Patent Documents or Published Foreign Patent Applications

Examiner Initial	Desig. ID	Document Number	Publication Date	Country or Patent Office	Class	Subclass	Translation	
							Yes	No
	2.							

Other Documents (include Author, Title, Date, and Place of Publication)

Examiner Initial	Desig. ID	Document
/A.B./	3.	Nuovo GJ, "In situ strand displacement amplification: an improved technique for the detection of low copy nucleic acids," Diagnostic Molecular Pathology, 2000, 9(4):195-202
/A.B./	4.	Zhu et al., "Engineering strand-specific DNA nicking enzymes from the type IIS restriction endonucleases Bsal, BsmBl, and BsmAl," J. Mol. Biol. 2004, 337:573-583
/A.B./	5.	Notice of Opposition in corresponding Application No. 08781827.4/2181196, dated August 7, 2015, pages 1-21

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Examiner Initial	Desig. ID	Document Number	Publication Date	Patentee	Class	Subclass	Filing Date If Appropriate
/A.B./	1.	2010/0204297	08/12/2010	Chen et al.			
/A.B./	2.	6,713,297	03/30/2004	McMillan et al.			
/A.B./	3.	6,977,148	12/20/2005	Dean et al.			
/A.B./	4.	2009/0111089	04/30/2009	Lindstrom et al.			
/A.B./	5.	2006/0257860	11/16/2006	Marlowe et al.			
/A.B./	6.	5,736,365	04/07/1998	Walker et al.			
/A.B./	7.	6,423,495	07/23/2002	Oultram et al.			
/A.B./	8.	2006/0160759	07/20/2006	Chen et al.			
/A.B./	9.	6,811,971	11/02/2004	Klepp et al.			
/A.B./	10.	5,614,387	03/25/1997	Shen et al.			
/A.B./	11.	5,834,254	11/10/1998	Shen et al.			
/A.B./	12.	6,077,669	06/20/2000	Little et al.			
/A.B./	13.	5,840,487	11/24/1998	Nadeau et al.			
/A.B./	14.	6,040,166	03/21/2000	Erlich et al.			
/A.B./	15.	6,929,915	08/16/2005	Benkovic et al.			
/A.B./	16.	2010/0184205	07/22/2010	Bentwich et al.			
/A.B./	17.	6,482,590	11/19/2002	Ullman et al.			
/A.B./	18.	6,767,724	07/27/2004	Lee et al.			
/A.B./	19.	6,335,164	01/01/2002	Kigawa et al.			
/A.B./	20.	6,660,475	12/09/2003	Jack et al.			
/A.B./	21.	7,297,485	11/20/2007	Bornarth et al.			

Foreign Patent Documents or Published Foreign Patent Applications								
Examiner Initial	Desig. ID	Document Number	Publication Date	Country or Patent Office	Class	Subclass	Translation	
							Yes	No
	22.							

Examiner Signature /Angela Bertagna/	Date Considered 02/05/2016
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(37 CFR §1.98(b))	Filing Date October 30, 2013	Group Art Unit 1637

Other Documents (include Author, Title, Date, and Place of Publication)		
Examiner Initial	Desig. ID	Document
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/A.B./	24.	Walker et al., "Multiplex strand displacement amplification (SDA) and detection of DNA sequences from *Mycobacterium tuberculosis* and other mycobacteria," Nucl. Acids Res. 22:2670-2677 (1994)
/A.B./	25.	Walker, "Empirical Aspects of Strand Displacement Amplification," PCR Methods and Appl., 3:1-6 (1993)
/A.B./	26.	Walker, et al., "Strand displacement amplification as an in vitro model for rolling-circle replication: Deletion formation and evolution during serial transfer," PNAS, 91:7937-7941 (1994)

Examiner Signature /Angela Bertagna/	Date Considered 02/05/2016
EXAMINER: Initials citation considered. Draw line through citation if not in conformance and not considered. Include copy of this form with next communication to applicant.	

Substitute Disclosure Form

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

First Named Inventor	:	Brian K. Maples	Art Unit	:	1637
Serial No.	:	14/067,620	Examiner	:	Angela Marie Bertagna
Filed	:	October 30, 2013	Conf. No.	:	4288
Title	:	NICKING AND EXTENSION AMPLIFICATION REACTION FOR THE EXPONENTIAL AMPLIFICATION OF NUCLEIC ACIDS			

MAIL STOP AMENDMENT
Commissioner for Patents
P.O. Box 1450
Alexandria, VA 22313-1450

INFORMATION DISCLOSURE STATEMENT

Please consider the references listed on the enclosed PTO-SB-08 or Disclosure Form. Foreign patent documents and non-patent literature are enclosed; cited U.S. patents and patent application publications will be provided on request.

This statement is being filed after a request for continued examination and before the mailing of a first action.

Apply any necessary charges or credits to deposit account 06-1050, referencing the above attorney docket number.

Please contact the undersigned if there are any questions regarding this Statement.

Respectfully submitted,

Date: February 2, 2016

/Ian J.S. Lodovice, Reg. No. 59,749/
Ian J. Lodovice
Reg. No. 59,749

Customer Number 26161
Fish & Richardson P.C.
Telephone: (617) 956-5972
Facsimile: (877) 769-7945

23485975.doc

Substitute Disclosure Form U.S. Department of Commerce Patent and Trademark Office	Attorney Docket No. 30171-0025002	Application No. 14/067,620
Information Disclosure Statement by Applicant (Use several sheets if necessary)	Applicant Ionian Technologies Inc.	
(37 CFR §1.98(b))	Filing Date October 30, 2013	Group Art Unit 1637

U.S. Patent Documents							
Examiner Initial	Desig. ID	Document Number	Publication Date	Patentee	Class	Subclass	Filing Date If Appropriate
	1.						

Foreign Patent Documents or Published Foreign Patent Applications								
Examiner Initial	Desig. ID	Document Number	Publication Date	Country or Patent Office	Class	Subclass	Translation	
							Yes	No
	2.							

Other Documents (include Author, Title, Date, and Place of Publication)		
Examiner Initial	Desig. ID	Document
	3.	Summons to Oral Proceedings in corresponding European Application No. 08781827.4, dated January 14, 2016, pages 1-17

Examiner Signature	Date Considered
EXAMINER: Initials citation considered. Draw line through citation if not in conformance and not considered. Include copy of this form with next communication to applicant.	

Substitute Disclosure Form

Electronic Acknowledgement Receipt

EFS ID:	24796052
Application Number:	14067620
International Application Number:	
Confirmation Number:	4288
Title of Invention:	Nicking and Extension Amplification Reaction for the Exponential Amplification of Nucleic Acids
First Named Inventor/Applicant Name:	Brian K. Maples
Customer Number:	26161
Filer:	Ian J.S. Lodovice/Mary Florczak
Filer Authorized By:	Ian J.S. Lodovice
Attorney Docket Number:	30171-0025002 / ITI-001
Receipt Date:	02-FEB-2016
Filing Date:	30-OCT-2013
Time Stamp:	15:05:49
Application Type:	Utility under 35 USC 111(a)

Payment information:

Submitted with Payment	no

File Listing:

Document Number	Document Description	File Name	File Size(Bytes)/ Message Digest	Multi Part /.zip	Pages (if appl.)
1	Information Disclosure Statement (IDS) Form (SB08)	301710025002IDS.pdf	174732 fcc9e91eb9b6944bfa443fb221451980de2a7091	no	2

Warnings:

Information:

This is not an USPTO supplied IDS fillable form

2	Non Patent Literature	30171Summons.pdf	5823866 4c93918b1361f1502bbd2a2187c21b8e1507269a	no	17
Warnings:					
Information:					
		Total Files Size (in bytes):	5998598		

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New Applications Under 35 U.S.C. 111
If a new application is being filed and the application includes the necessary components for a filing date (see 37 CFR 1.53(b)-(d) and MPEP 506), a Filing Receipt (37 CFR 1.54) will be issued in due course and the date shown on this Acknowledgement Receipt will establish the filing date of the application.

National Stage of an International Application under 35 U.S.C. 371
If a timely submission to enter the national stage of an international application is compliant with the conditions of 35 U.S.C. 371 and other applicable requirements a Form PCT/DO/EO/903 indicating acceptance of the application as a national stage submission under 35 U.S.C. 371 will be issued in addition to the Filing Receipt, in due course.

New International Application Filed with the USPTO as a Receiving Office
If a new international application is being filed and the international application includes the necessary components for an international filing date (see PCT Article 11 and MPEP 1810), a Notification of the International Application Number and of the International Filing Date (Form PCT/RO/105) will be issued in due course, subject to prescriptions concerning national security, and the date shown on this Acknowledgement Receipt will establish the international filing date of the application.

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

First Named Inventor	:	Brian K. Maples	Art Unit	:	1637
Serial No.	:	14/067,620	Examiner	:	Angela Marie Bertagna
Filed	:	October 30, 2013	Conf. No.	:	4288
Title	:	NICKING AND EXTENSION AMPLIFICATION REACTION FOR THE EXPONENTIAL AMPLIFICATION OF NUCLEIC ACIDS			

MAIL STOP AMENDMENT
Commissioner for Patents
P.O. Box 1450
Alexandria, VA 22313-1450

INFORMATION DISCLOSURE STATEMENT

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This statement is being filed after a request for continued examination and before the mailing of a first action.

Apply any necessary charges or credits to deposit account 06-1050, referencing the above attorney docket number.

Please contact the undersigned if there are any questions regarding this Statement.

Respectfully submitted,

Date: January 22, 2016

/Ian J.S. Lodovice, Reg. No. 59,749/
Ian J. Lodovice
Reg. No. 59,749

Customer Number 26161
Fish & Richardson P.C.
Telephone: (617) 956-5972
Facsimile: (877) 769-7945

23482413.doc

Substitute Disclosure Form U.S. Department of Commerce Patent and Trademark Office	Attorney Docket No. 30171-0025002	Application No. 14/067,620
Information Disclosure Statement by Applicant (Use several sheets if necessary)	Applicant Ionian Technologies Inc.	
(37 CFR §1.98(b))	Filing Date October 30, 2013	Group Art Unit 1637

U.S. Patent Documents							
Examiner Initial	Desig. ID	Document Number	Publication Date	Patentee	Class	Subclass	Filing Date If Appropriate
	1.	2010/0204297	08/12/2010	Chen et al.			
	2.	6,713,297	03/30/2004	McMillan et al.			
	3.	6,977,148	12/20/2005	Dean et al.			
	4.	2009/0111089	04/30/2009	Lindstrom et al.			
	5.	2006/0257860	11/16/2006	Marlowe et al.			
	6.	5,736,365	04/07/1998	Walker et al.			
	7.	6,423,495	07/23/2002	Oultram et al.			
	8.	2006/0160759	07/20/2006	Chen et al.			
	9.	6,811,971	11/02/2004	Klepp et al.			
	10.	5,614,387	03/25/1997	Shen et al.			
	11.	5,834,254	11/10/1998	Shen et al.			
	12.	6,077,669	06/20/2000	Little et al.			
	13.	5,840,487	11/24/1998	Nadeau et al.			
	14.	6,040,166	03/21/2000	Erlich et al.			
	15.	6,929,915	08/16/2005	Benkovic et al.			
	16.	2010/0184205	07/22/2010	Bentwich et al.			
	17.	6,482,590	11/19/2002	Ullman et al.			
	18.	6,767,724	07/27/2004	Lee et al.			
	19.	6,335,164	01/01/2002	Kigawa et al.			
	20.	6,660,475	12/09/2003	Jack et al.			
	21.	7,297,485	11/20/2007	Bornarth et al.			

Foreign Patent Documents or Published Foreign Patent Applications								
Examiner Initial	Desig. ID	Document Number	Publication Date	Country or Patent Office	Class	Subclass	Translation Yes	Translation No
	22.							

Examiner Signature	Date Considered
EXAMINER: Initials citation considered. Draw line through citation if not in conformance and not considered. Include copy of this form with next communication to applicant.	

Substitute Disclosure Form

Substitute Disclosure Form U.S. Department of Commerce Patent and Trademark Office	Attorney Docket No. 30171-0025002	Application No. 14/067,620
Information Disclosure Statement by Applicant (Use several sheets if necessary)	Applicant Ionian Technologies Inc.	
(37 CFR §1.98(b))	Filing Date October 30, 2013	Group Art Unit 1637

Other Documents (include Author, Title, Date, and Place of Publication)		
Examiner Initial	Desig. ID	Document
	23.	Walker et al., "Strand displacement amplification- an iso-thermal, in vitro DNA amplification technique," Nucl. Acids Res. 20:1691-1696 (1992)
	24.	Walker et al., "Multiplex strand displacement amplification (SDA) and detection of DNA sequences from *Mycobacterium tuberculosis* and other mycobacteria," Nucl. Acids Res. 22:2670-2677 (1994)
	25.	Walker, "Empirical Aspects of Strand Displacement Amplification," PCR Methods and Appl., 3:1-6 (1993)
	26.	Walker, et al., "Strand displacement amplification as an in vitro model for rolling-circle replication: Deletion formation and evolution during serial transfer," PNAS, 91:7937-7941 (1994)

Examiner Signature	Date Considered
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Substitute Disclosure Form

Electronic Acknowledgement Receipt

EFS ID:	24704031
Application Number:	14067620
International Application Number:	
Confirmation Number:	4288
Title of Invention:	Nicking and Extension Amplification Reaction for the Exponential Amplification of Nucleic Acids
First Named Inventor/Applicant Name:	Brian K. Maples
Customer Number:	26161
Filer:	Ian J.S. Lodovice/Mary Florczak
Filer Authorized By:	Ian J.S. Lodovice
Attorney Docket Number:	30171-0025002 / ITI-001
Receipt Date:	22-JAN-2016
Filing Date:	30-OCT-2013
Time Stamp:	16:55:12
Application Type:	Utility under 35 USC 111(a)

Payment information:

Submitted with Payment	no

File Listing:

Document Number	Document Description	File Name	File Size(Bytes)/ Message Digest	Multi Part /.zip	Pages (if appl.)
1	Information Disclosure Statement (IDS) Form (SB08)	301710025002IDS.pdf	196989 7a87ab097d32ca00d08d9aea41960bde72362a87	no	3

Warnings:

Information:

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2	Non Patent Literature	walker.pdf	1347184 035b1267d5ac4fbb2e7ee435e1051aefe39a4b73	no	6
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Information:					
3	Non Patent Literature	Walker2.pdf	2209065 fc15331b61bda6109b489daa55c7aa318157e4f8	no	8
Warnings:					
Information:					
4	Non Patent Literature	Walker3.pdf	1373342 97156caa3245d1813d8d24d0e9207e25033b2f0e	no	7
Warnings:					
Information:					
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Warnings:					
Information:					
		Total Files Size (in bytes):	6527902		

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New Applications Under 35 U.S.C. 111
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First Named Inventor	:	Brian K. Maples	Art Unit	:	1637
Serial No.	:	14/067,620	Examiner	:	Angela Marie Bertagna
Filed	:	October 30, 2013	Conf. No.	:	4288
Title	:	NICKING AND EXTENSION AMPLIFICATION REACTION FOR THE EXPONENTIAL AMPLIFICATION OF NUCLEIC ACIDS			

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Please contact the undersigned if there are any questions regarding this Statement.

Respectfully submitted,

Date: November 25, 2015

/Ian J.S. Lodovice, Reg. No. 59,749/
Ian J. Lodovice
Reg. No. 59,749

Customer Number 26161
Fish & Richardson P.C.
Telephone: (617) 956-5972
Facsimile: (877) 769-7945

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Substitute Disclosure Form	U.S. Department of Commerce Patent and Trademark Office	Attorney Docket No. 30171-0025002	Application No. 14/067,620
Information Disclosure Statement by Applicant (Use several sheets if necessary)		Applicant Ionian Technologies Inc.	
(37 CFR §1.98(b))		Filing Date October 30, 2013	Group Art Unit 1637

U.S. Patent Documents

Examiner Initial	Desig. ID	Document Number	Publication Date	Patentee	Class	Subclass	Filing Date If Appropriate
	1.	2008/0096257	04/24/2008	Yao et al.			

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Examiner Initial	Desig. ID	Document Number	Publication Date	Country or Patent Office	Class	Subclass	Translation	
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	3.	Nuovo GJ, "In situ strand displacement amplification: an improved technique for the detection of low copy nucleic acids," Diagnostic Molecular Pathology, 2000, 9(4):195-202
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Application Number:	14067620
International Application Number:	
Confirmation Number:	4288
Title of Invention:	Nicking and Extension Amplification Reaction for the Exponential Amplification of Nucleic Acids
First Named Inventor/Applicant Name:	Brian K. Maples
Customer Number:	26161
Filer:	Ian J.S. Lodovice/Mary Florczak
Filer Authorized By:	Ian J.S. Lodovice
Attorney Docket Number:	30171-0025002 / ITI-001
Receipt Date:	25-NOV-2015
Filing Date:	30-OCT-2013
Time Stamp:	16:46:17
Application Type:	Utility under 35 USC 111(a)

Payment information:

Submitted with Payment	no

File Listing:

Document Number	Document Description	File Name	File Size(Bytes)/ Message Digest	Multi Part /.zip	Pages (if appl.)
1	Information Disclosure Statement (IDS) Form (SB08)	301710025002IDS.pdf	177072 800ea17412238bdb35e4f311f3deb090aea16c29	no	2

Warnings:

Information:

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2	Non Patent Literature	NoticeOpposition.pdf	3588036 1282f0ffbc655fe1c07dcd24bed3c52b7e03e922	no	21
Warnings:					
Information:					
3	Non Patent Literature	Nuovo.pdf	607054 0bf7ee338f65ed7964f971d503e56aedc12e4def	no	8
Warnings:					
Information:					
4	Non Patent Literature	Zhu.pdf	1651915 4fd4761cf63726614b05b1491cf726e331a9bf43	no	11
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IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

First Named Inventor	:	Brian K. Maples	Art Unit	:	1637
Serial No.	:	14/067,620	Examiner	:	Olayinka A. Oyeyemi
Filed	:	October 30, 2013	Conf. No.	:	4288
Title	:	NICKING AND EXTENSION AMPLIFICATION REACTION FOR THE EXPONENTIAL AMPLIFICATION OF NUCLEIC ACIDS			

Mail Stop Amendment
Commissioner for Patents
P.O. Box 1450
Alexandria, VA 22313-1450

INTERVIEW SUMMARY

Applicant thanks Examiner Bertagna for the courtesy of a telephonic interview with Applicant's representatives Ian Lodovice, Rich Roth, and Belinda Lew on September 15, 2015. During the interview, the participants discussed the response filed July 27, 2015 and arguments regarding the prior art rejections over Wick (US 6,063,604), Kong (US 6,191,267) and Yao (US 20090092967), all of which or of record. Applicant acknowledges and thanks Examiner Bertagna for the Applicant-Initiated Interview Summary dated September 21, 2015.

Respectfully submitted,

Date: October 21, 2015______________

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23450691.doc

Electronic Acknowledgement Receipt

EFS ID:	23849484
Application Number:	14067620
International Application Number:	
Confirmation Number:	4288
Title of Invention:	Nicking and Extension Amplification Reaction for the Exponential Amplification of Nucleic Acids
First Named Inventor/Applicant Name:	Brian K. Maples
Customer Number:	26161
Filer:	Ian J.S. Lodovice/Mary Florczak
Filer Authorized By:	Ian J.S. Lodovice
Attorney Docket Number:	30171-0025002 / ITI-001
Receipt Date:	21-OCT-2015
Filing Date:	30-OCT-2013
Time Stamp:	14:38:05
Application Type:	Utility under 35 USC 111(a)

Payment information:

Submitted with Payment	no

File Listing:

Document Number	Document Description	File Name	File Size(Bytes)/ Message Digest	Multi Part /.zip	Pages (if appl.)
1	Applicant summary of interview with examiner	301710025002IS.pdf	64241 c70ad9f6522f4e4941d186c7291c39da69a16ef4	no	1

Warnings:

Information:

Total Files Size (in bytes):	64241

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IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

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MAIL STOP AMENDMENT
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Alexandria, VA 22313-1450

INFORMATION DISCLOSURE STATEMENT

Please consider the references listed on the enclosed PTO-SB-08 or Disclosure Form. Foreign patent documents are enclosed.

This statement is being filed after a request for continued examination and before the mailing of a first action.

Apply any necessary charges or credits to deposit account 06-1050, referencing the above attorney docket number.

Please contact the undersigned if there are any questions regarding this Statement.

Respectfully submitted,

Date: September 30, 2015____________

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23442751.doc

Substitute Disclosure Form U.S. Department of Commerce Patent and Trademark Office	Attorney Docket No. 30171-0025002	Application No. 14/067,620
Information Disclosure Statement by Applicant (Use several sheets if necessary)	Applicant Ionian Technologies Inc.	
(37 CFR §1.98(b))	Filing Date October 30, 2013	Group Art Unit 1637

U.S. Patent Documents							
Examiner Initial	Desig. ID	Document Number	Publication Date	Patentee	Class	Subclass	Filing Date If Appropriate
	1.						

Foreign Patent Documents or Published Foreign Patent Applications								
Examiner Initial	Desig. ID	Document Number	Publication Date	Country or Patent Office	Class	Subclass	Translation	
							Yes	No
	2.	WO2007096702	08/30/2007	WIPO				

Other Documents (include Author, Title, Date, and Place of Publication)		
Examiner Initial	Desig. ID	Document
	3.	

Examiner Signature	Date Considered
EXAMINER: Initials citation considered. Draw line through citation if not in conformance and not considered. Include copy of this form with next communication to applicant.	

Substitute Disclosure Form

(12) INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(19) World Intellectual Property Organization
International Bureau

(43) International Publication Date
30 August 2007 (30.08.2007)

PCT

(10) International Publication Number
WO 2007/096702 A2

(51) International Patent Classification: Not classified

(21) International Application Number: PCT/IB2006/004113

(22) International Filing Date: 25 July 2006 (25.07.2006)

(25) Filing Language: English

(26) Publication Language: English

(30) Priority Data:
60/702,533 25 July 2005 (25.07.2005) US
60/728,424 18 October 2005 (18.10.2005) US

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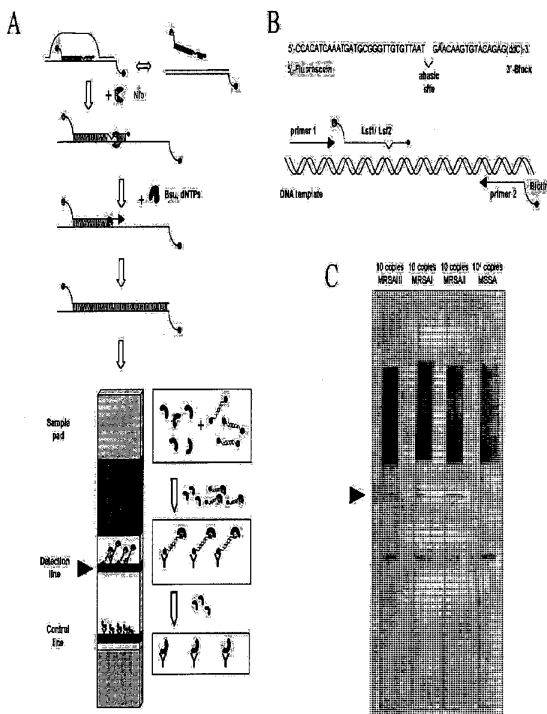
(81) Designated States (*unless otherwise indicated, for every kind of national protection available*): AE, AG, AL, AM, AT, AU, AZ, BA, BB, BG, BR, BW, BY, BZ, CA, CH, CN, CO, CR, CU, CZ, DE, DK, DM, DZ, EC, EE, EG, ES, FI, GB, GD, GE, GH, GM, HN, HR, HU, ID, IL, IN, IS, JP, KE, KG, KM, KN, KP, KR, KZ, LA, LC, LK, LR, LS, LT, LU, LV, LY, MA, MD, MG, MK, MN, MW, MX, MZ, NA, NG, NI, NO, NZ, OM, PG, PH, PL, PT, RO, RS, RU, SC, SD, SE, SG, SK, SL, SM, SY, TJ, TM, TN, TR, TT, TZ, UA, UG, US, UZ, VC, VN, ZA, ZM, ZW.

(84) Designated States (*unless otherwise indicated, for every kind of regional protection available*): ARIPO (BW, GH, GM, KE, LS, MW, MZ, NA, SD, SL, SZ, TZ, UG, ZM, ZW), Eurasian (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European (AT, BE, BG, CH, CY, CZ, DE, DK, EE, ES, FI, FR, GB, GR, HU, IE, IS, IT, LT, LU, LV, MC, NL, PL, PT, RO, SE, SI, SK, TR), OAPI (BF, BJ, CF, CG, CI, CM, GA, GN, GQ, GW, ML, MR, NE, SN, TD, TG).

[Continued on next page]

(54) Title: METHODS FOR MULTIPLEXING RECOMBINASE POLYMERASE AMPLIFICATION



(57) Abstract: This disclosure provides for methods and reagents for rapid multiplex RPA reactions and improved methods for detection of multiplex RPA reaction products. In addition, the disclosure provides new methods for eliminating carryover contamination between RPA processes.

WO 2007/096702 A2

Published:
— *without international search report and to be republished upon receipt of that report*

For two-letter codes and other abbreviations, refer to the "Guidance Notes on Codes and Abbreviations" appearing at the beginning of each regular issue of the PCT Gazette.

METHODS FOR MULTIPLEXING RECOMBINASE POLYMERASE AMPLIFICATION

RELATED APPLICATIONS

This application claims the benefit of priority from U.S. Appl. 60/702,533 filed July 25, 2005 and U.S. Appl. 60/728,424 filed October 18, 2005.

BACKGROUND

Recombinase Polymerase Amplification (RPA) is a DNA amplification process that utilizes enzymes to match synthetic oligonucleotide primers to their complementary partners in duplex DNA. (Armes and Stemple, US patent Appl. 60/358,563 filed Feburuary 21, 2002). RPA depends upon components of the cellular DNA replication and repair machinery. The notion of employing some of this machinery for in vitro DNA amplification has existed for some time (Zarling et al. US patent 5,223,414), however the concept has not transformed to a working technology until recently as, despite a long history of research in the area of recombinase function involving principally the E.coli recA protein, in vitro conditions permitting sensitive amplification of DNA have only recently been determined (Piepenburg et al. US patent application 10/931,916 filed September 1, 2004, also Piepenburg et al., PlosBiology 2006).

RPA offers a number of advantages over traditional methods of DNA amplification. These advantages include the lack of a need for any initial thermal or chemical melting, the ability to operate at low constant temperatures without a need for absolute temperature control, as well as the observation that complete reactions (lacking target) can be stored in a dried condition. These characteristics demonstrate that RPA is a uniquely powerful tool for developing portable, accurate, and instrument-free nucleic acid detection tests.

BRIEF DESCRIPTION OF THE INVENTION

The present invention relates to methods of nucleic acid amplification which include novel recombinase polymerase amplification (RPA) protocols for rapid and efficient amplification of nucleic acids in a process that can be easily multiplexed.

One embodiment of the invention is directed to a method wherein a plurality of RPA which can be performed simultaneously in a single reaction (in a single tube) and wherein the results may be detected simultaneously. The single RPA reaction is described first below and methods of multiplexing said reaction is described second.

One aspect of the invention is directed to methods of RPA which generates easily detectable amplimers (an amplified nucleic acid which is the product of an RPA reaction). The RPA process amplified a double stranded target nucleic acid molecule comprising a first and a second strand of DNA. Step (a) involves contacting a recombinase agent with a first and a second nucleic acid primer and a third extension blocked primer which comprises one or more noncomplementary or modified internal residue to form a first, second and third nucleoprotein primer. Step (b) involves contacting the first and second nucleoprotein primers to said double stranded target nucleic acid thereby forming a first double stranded structure between said first nucleoprotein primer and said first strand of DNA at a first portion of said first strand (forming a D loop) and a second double stranded structure between said second nucleoprotein primer and said second strand of DNA at a second portion of said second strand (forming a D loop) such that the 3' ends of said first nucleoprotein primer and said first nucleoprotein primer are oriented toward each other on the same target nucleic acid molecule with a third portion of target nucleic acid between said 3' ends; Step (c) involves extending the 3' end of said first nucleoprotein primer and second nucleoprotein primer with one or more polymerases and dNTPs to generate a first amplified target nucleic acid with an internal region comprising the third portion of nucleic acid. Step (d) involves contacting said amplified target nucleic acid to said third nucleoprotein primer to form a third double stranded structure at the third portion of said amplified target nucleic acid (forming a D loop) in the presences of a nuclease; wherein said nuclease specifically cleaves said noncomplementary internal residue only after the formation of said third double stranded structure to form a third 5' primer and a third 3' extension blocked primer. Step (d) involves extending the 3' end of said third 5' primer with one or more polymerase and dNTP to generate a second double stranded amplified nucleic acid which comprises said first nucleic acid primer and said third 5' primer. The RPA reaction is continued until a desired degree of

the second double stranded amplified nucleic acid is reached. It should be noted that this process, along with any related embodiments, may be used for multiplex RPA reaction (described below).

The recombinase agent may be, for example, uvsX, RecA and functional analogs thereof. Further, the RPA reaction may be performed in the presence of uvxY, gp32, single strand binding proteins and other usual RPA reagents. Methods for performing RPA are disclosed, for example, in U.S. Appl. 60/358,563 filed February 21, 2002, U.S. Appl. 10/371,641, filed February 21, 2003, 2003, U.S. pat. appl. 10/931,916 filed September 1, 2004 and PCT/IB2005/001560 (WO2005/118853) filed April 11, 2005.

The nuclease used in this RPA reaction should specifically cleave the noncomplementary residue or the modified internal residue preferentially when the third extension blocked primer is hybridized to a DNA to form a double stranded structure. It is preferred that the nuclease do not cleave the noncomplementary residue or the modified internal residue when the extension blocked primer is in single stranded form - regardless of whether the primer is attached to recombinase or SSB. In a preferred embodiment, the nuclease is a DNA glycosylase or AP endonuclease. If the modified internal residue is a uracil or inosine, the preferred nuclease is uracil glycosylase or hypoxanthine-DNA glycosylase respectively. The nuclease may recognize the noncomplementary base by nature of a mismatch which forms a region of noncomplementary residues (i.e., a bubble) in an otherwise double stranded structure. In this case, the nuclease recognizes a base mismatch between the noncomplementary residues and cleaves primer at the noncomplementary base.

The nuclease used in any of the processes of the invention may be a DNA glycosylase or an AP endonuclease. The nuclease may function by recognizing a base mismatch between said first extension blocked primer and said target nucleic acid and cleaving the extension blocked primer at the base mismatch without cleaving the target nucleic acid. The nuclease, alternatively, may recognize a damaged residue, an abasic site or abasic site mimic, or any other modification which may be incorporated into synthetic oligonucleotides. The nuclease may be, for example, fpg, Nth, MutY, MutS, MutM, E. coli. MUG, human MUG, human Oggl, a vertebrate Nei-like (Neil) glycosylases, Nfo, exonuclease III, uracil glycosylase, hypoxanthine-DNA and functional analogs and homologs thereof. The functional analogs and homologs may be of any mammalian, bacterial or viral original. As additional examples, if the modified base is inosine, the nuclease may be hypoxanthine-DNA glycosylase; if the modified base is uracil, the nuclease may be uracil glycosylase. In a preferred embodiment,

these nucleases may be from E. coli. In a preferred embodiment, the nuclease is E. coli Nfo or E. coli exonuclease III and the modified internal residue is a tetrahydrofuran residue or a linker group. A 'linker' (also called a carbon linker or 'spacer') is a carbon-containing chain which is used to join the 3' position of one sugar to the (usually) 5' position of another. Common spacers may comprise about 3, 6, 9, 12 or 18 carbon chains although it may be of any number of carbon chains. Carbon-oxygen-carbon linkages are common in these spacers, presumably to reduce hydrophobicity. Nfo and exonuclease III (and homologs) can recognize the sugar 3'-O-C linkage on the 3' end of a nucleotide linked to a spacer and cleave it. See, for example, C18 spacer (18 - O - Dimethoxytritylhexaethyleneglycol, 1 - [(2 - cyanoethyl) - (N, N - diisopropyl)] - phosphoramidite (Glen Research, Sterling, VA, USA, cat#10-1918-90).

As used herein, an "abasic residue" in an oligonucleotide refers to a molecular fragment (MF) within an oligonucleotide chain where the molecular fragment approximates the length of a ribofuranose or a deoxyribofuranose sugar in such a way that bases adjacent to the molecular fragment are separated from one another by the same, or effectively the same, distance as if a ribofuranose or a deoxyribofuranose sugar of any of A, G, C, T, or U were present in place of the abasic residue. The abasic residue may incorporate a ribofuranose or deoxyribofuranose ring as in native A, G, C, T, or U. However, the abasic residue does not contain a base or other molecule that can interact with the base on the opposite strand of a duplex which is formed with the abasic residue-containing oligonucleotide. Thus, an abasic residue may be an apurine or apyrimidine structure, a base analog, or an analogue of a phosphate backbone. The abasic substitution may also consist of a backbone of N-(2-aminoethyl)-glycine linked by amide bonds. In a preferred embodiment, the abasic residue is tetrahydrofuran or D-spacer (a type of tetrahydrofuran). Both a D-spacer and tetrahydrofuran effectively are a deoxyribose sugar in which both the 1' and 2' position lack OH residues. Normally the 1' position of a true abasic residue in DNA would have a hydroxyl in the position where the base is normally attached, however this is unstable as the ring form interconverts with an open-ring aldehyde form (see below) which can then degrade by the process of beta-elimination. Removal of this hydroxyl leads to a stable form readily synthesized into oligonucleotides. Tetrahydrofuran-type abasic sites and their use as abasic residues are known. The tetrahydrofuran may be placed into oligonucleotides during synthesis by ordering reagents from Glen Research (Sterling, Virginia, USA).

The one or more noncomplementary or modified internal residue is internal because it is not the 5' most or 3' most residue of the first extension blocked primer. In a preferred embodiment, the one or more noncomplementary internal residue is at least 10 residues away from the 5' or 3' residue of a primer. In a more preferred embodiment, the one or more noncomplementary internal residue is at least 15, or at least 20 residues away from the 5' or 3' residue of a primer.

The one or more noncomplementary internal residue may be introduced by synthesizing an oligonucleotide primer with one or more noncomplementary residue. A noncomplementary residue is any residue that does not form a Watson Crick base pair (hydrogen bond) with its corresponding residue in a double stranded structure. For example, if a "T" at a particular location is needed to form a Watson-Crick base pair between a primer and a target nucleic acid, the use of an "A" would cause the "A" to be non complementary. As a further example, each of the middle bases in the following double stranded structure is a noncomplementary base.

```
primer     aaaaa (SEQ ID NO:1)
           || ||
target     ttatt (SEQ ID NO:2)

primer     aagaa (SEQ ID NO:3)
           || ||
target     ttatt (SEQ ID NO:4)

primer     aacaa (SEQ ID NO:5)
           || ||
target     ttatt (SEQ ID NO:6)
```

It is known that the presence of noncomplementary residues in a double stranded nucleic acid will produce a bubble within the double stranded nucleic acid. While one noncomplementary or modified internal residue is sufficient for functioning with the methods of the invention, more than one noncomplementary or modified internal residues may be used. When more than one is used, they may adjacent to each other on an oligonucleotide or they may be separated. It should be noted that if the nuclease cleaves the target nucleic acid at the mismatch or noncomplementary location, the target DNA is repaired rapidly by dNTP

and polymerase using the primer as a template. Because of this, this reaction would not affect the processes of this disclosure.

The one or more noncomplementary internal residue of the first extension blocked primer may be a modified internal residue. The modified internal residue may be any chemical structure (residue) that cannot form a Watson-Crick base pairing structure with its corresponding base in a double stranded nucleic acid structure. If more than one noncomplementary internal residue is used, they can be a mixture of noncomplementary internal residues or modified internal residues. The term “modified internal residue,” also includes, at least, any residue not normally found in DNA - that is any residue which is not an “A”, “G”, “C” or “T” such as, for example uracil or inosine.

The modified internal residue may be inosine, uracil, 8-oxoguanine, thymine glycol, or an abasic site mimic. Preferred abasic site mimics include a tetrahydrofuran residue or D-spacer (which can be produced as a product of employing a 5' - O - Dimethoxytrityl-1',2' - Dideoxyribose-3' - [(2-cyanoethyl) - (N,N-diisopropyl)]-phosphoramidite during oligonucleotide synthesis.

The extension blocked primer is blocked at its 3’ end so that it cannot normally be elongated by polymerase and dNTP even in the presence of a complimentary template. Methods of blocking a primer are well known and include, at least, the inclusion of a blocked 3’ nucleotide. The blocked 3’ nucleotide may contain, for example, a blocking group that prevents polymerase extension. Generally, the blocking groups are attached to the 3’ or 2’ site of the 3’ sugar residue but other locations of attachments are possible. One of the most common 3’ blocking methods is to place a dideoxy sugar at the 3’ end of an oligonucleotide. The blocking group may be, for example, a detectable label.

A detectable label is defined as any moiety that may be detected using current methods. These labels include, at least, a fluorophore (also called a fluorescent molecule, fluorochrome), an enzyme, a quencher, an enzyme inhibitor, a radioactive label, a member of a binding pair, a digoxygenin residue, a peptide, and a combination thereof.

"A member of a binding pair" is meant to be one of a first and a second moiety, wherein said first and said second moiety have a specific binding affinity for each other. Suitable binding pairs for use in the invention include, but are not limited to, antigens/antibodies (for example, digoxigenin/anti-digoxigenin, dinitrophenyl (DNP)/anti-DNP, dansyl-X-anti-dansyl, Fluorescein/anti-fluorescein, lucifer yellow/anti-lucifer yellow,

peptide/anti-peptide, ligand/receptor and rhodamine/anti-rhodamine), biotin/avidin (or biotin/streptavidin) and calmodulin binding protein (CBP)/calmodulin. Other suitable binding pairs include polypeptides such as the FLAG-peptide (DYKDDDDK; SEQ ID NO:7) [Hopp et al., BioTechnology, 6:1204 1210 (1988)]; the KT3 epitope peptide (Martin et al., Science 255:192 194 (1992)); tubulin epitope peptide (Skinner et al., J. Biol. Chem 266:15163 15166 (1991)); and the T7 gene 10 protein peptide tag (Lutz-Freyermuth et al., Proc. Natl. Acad. Sci. USA, 87:6393 6397 (1990)) and the antibodies each thereto. Generally, in a preferred embodiment, the smaller of the binding pair partners serves as the detectable label, as steric considerations may be important. In addition to the above, any of the nucleic acid and nucleotides of the RPA reaction may be labeled with a detectable label.

In any of the RPA processes of the invention where a detectable label is used, the detectable label may be used to monitor the progress (the production of amplimers) of the RPA reaction. In one aspect, if the primers are labeled, monitoring may involve detecting a label in an amplimer. Since amplimers would be expected to be larger than the primers used, detection may involve, for example gel electrophoresis and the detection of the proper sized amplimer. Alternatively, labeled amplimers may be separated by labeled primers by a more rapid process such as column chromatography (including spin columns, push columns and the like). Since the RPA methods of the invention has high specificity and low artifact production (high signal to noise), monitoring may involve performing RPA using nucleotides attached to detectable labels and measuring the amount of labels attached to high molecular weight nucleic acid (e.g., nucleic acid of more than 100 bases in length). For example, radioactive dNTPs may be used and the progress of the RPA reaction may be monitored by following the incorporation of radiation into high molecular weight DNA. Techniques that monitor incorporation of nucleotides into high molecular weight DNA include gel electrophoresis, size exclusion column (e.g., conventional, spin and push columns) and acid precipitation.

If the first nucleic acid primer and the third 5' primer are each labeled with a different detectable label, then the amplified product (the second double stranded amplified nucleic acid) will be the only nucleic acid species with both labels. This double labeled nucleic acid species may be detected by a variety of means. In one preferred method, the amplified product may be detected using a flow strip. In one preferred embodiment, one detectable label produces a color and the second label is an epitope which is recognized by an immobilized antibody. A product containing both labels will attach to an immobilized

antibody and produce a color at the location of the immobilized antibody. An assay based on this detection method may be, for example, a flow strip (dip stick) which can be applied to the whole RPA reaction. A positive amplification will produce a band on the flow strip while a negative amplification would not produce any color band.

It should be noted that this RPA amplification process using 3 primers may be multiplexed (referred to herein as multiplex RPA). That is, multiple RPA process using 3 primers, as discussed above, may be performed in the same reaction (tube). Multiplex RPA may be performed with one or more target nucleic acids. Each process is performed with a different combination of first and second nucleic acid primers which is specific for a different region of one or more target nucleic acids. In a preferred embodiment, when multiple RPA processes are performed in the same reaction, each RPA process uses a first nucleic acid with the same label but not necessarily the same sequence. Further, each process uses the same third extension blocked primer with a second detectable label. In this way, by measuring the accumulation of double stranded nucleic acid product with both the first detectable label and the second detectable label, the cumulative amplification of each RPA process may be measured.

Multiplexed RPA is useful for many purposes. For example, multiple pathogens may share a common nucleic acid sequence that is too small for direct amplification by RPA. Furthermore, the common nucleic acid sequence have different flanking sequence in each organism so that a single set of RPA primers cannot be designed to amplify this common nucleic acid sequence in multiple organisms. Using the process of multiplex RPA as described above, a plurality of combination of RPA primers may be used in one reaction, wherein each combination would amplify the common nucleic acid sequence in one organism and this common nucleic acid sequence would be concomitantly amplified by the common third primer (third extension blocked primer). Multiplex RPA with primer combinations designed to detect multiple pathogens, may be used for example, in an assay to detect methicillin resistant S. aureus strains by amplifying and detecting a common sequence (e.g., mec2) in each strain. By using the multiplexed RPA of the invention, a plurality of loci (DNA sequences) may be detected by concurrent RPA amplification. In a preferred embodiment, at least 2 simultaneous RPA are performed in an RPA. In a more preferred embodiment, at least 3, at least 5, at least 7 or at least 10 RPA reactions may be performed in the same tube.

Thus, another aspect of the invention is directed to a multiplex method of RPA comprising the steps of performing more than one RPA process in one reaction. Each individual reaction is performed as described above for RPA using 3 primers. Briefly, each reaction involves the steps of (a1) contacting a recombinase agent with a first and a second nucleic acid primer and a third extension blocked primer which comprises a noncomplementary or modified internal residue to form a first, second and third nucleoprotein primer; (a2) contacting the first and second nucleoprotein primers to said double stranded target nucleic acid thereby forming a first double stranded structure between said first nucleoprotein primer and said first strand of DNA at a first portion of said first strand and a second double stranded structure between said second nucleoprotein primer and said second strand of DNA at a second portion of said second strand such that the 3' ends of said first nucleoprotein primer and said first nucleoprotein primer are oriented toward each other on the same target nucleic acid molecule with a third portion of nucleic acid between said 3' ends; (a3) extending the 3' end of said first nucleoprotein primer and second nucleoprotein primer with one or more polymerases and dNTPs to generate a first amplified target nucleic acid with an internal region comprising the third portion of nucleic acid; (a4) contacting said amplified target nucleic acid to said third nucleoprotein primer to form a third double stranded structure at the third portion of said amplified target nucleic acid in the presences of a nuclease; wherein said nuclease specifically cleaves said noncomplementary or modified internal residue only after the formation of said third double stranded structure to form a third 5' primer and a third 3' extension blocked primer; (a5) extending the 3' end of said third 5' primer to generate a second double stranded amplified nucleic acid which comprises said first nucleic acid primer and said third 5' primer; (a6) continuing the reaction through repetition of (a2) and (a5) until a desired degree of the second double stranded amplified nucleic acid is reached. In this process, each RPA process is performed with a different combination of first and second nucleic acid primers but each process is performed with the same third extension blocked primer.

It should be noted that while each RPA process will have a different combination of first and second nucleic acid primers, primers can still be shared between RPA processes. For example, RPA process 1 may use primers 1 and 2 while RPA process 2 may use primers 2 and 3. Thus, RPA process 1 and RPA process 2 share the same primer (primer 2).

In any RPA process that involves an extension blocked primer (e.g., the third extension blocked primer) the primer may further comprises one or more detectable labels

and the progress of the RPA may be monitored a second way by monitoring the detectable label on this primer. The detectable label may be a fluorophore, an enzyme, a quencher, an enzyme inhibitor, a radioactive label, one member of a binding pair and a combination of thereof. Where a fluorophore or quencher is used, the attachment may be by a fluorophore-dT amidite residue or a quencher-dT amidite residue.

In a preferred embodiment, the third extension blocked primer comprises a fluorophore and a quencher. The fluorophore and quencher are separated by between 0 to 2 bases, 0 to 5 bases, 0 to 8 bases or 0 to 10 bases, 3 to 5 bases, 6 to 8 bases, or 8 to 10 bases. In addition, the fluorophore and quencher may be separated by a greater distance when the extension blocked primer is unhybridized than when the extension blocked primer is hybridized to the target nucleic acid. Furthermore, the fluorophore or quencher may be attached to the noncomplementary or modified internal residue as long as the fluorophore and quencher are separated following cleavage of the modified internal base by the nuclease. Preferred fluorophores include fluorescein, FAM, TAMRA and preferred quenchers include a dark quencher (e.g., Dark Quencher 1, Dark Quencher 2, Black Hole Quencher 1 and Black Hole Quencher 2).

One advantage of the methods of this RPA process is that it can be performed at a low temperature such as between 14°C and 21°C, between 21°C and 25°C, between 25°C and 30°C, between 30°C and 37°C or between, 40°C and 43°C. Under these temperature conditions, the reaction are accelerated in the presence of 1% to 12% PEG such as between 6% to 8% PEG.

Another advantage of using extension blocked primers, for any of the methods of the invention, is that the progress of the reaction may be monitored in real time. Monitoring may involve, for example, measuring fluorescence in the RPA reaction. In this method, the fluorophore and quencher are located at a sufficiently close distance (less than 10 residues apart, as disclosed in this specification) on the primer such that the quencher prevents fluorescence from the fluorophore. However, as the third extension blocked primer is cleaved by the nuclease, the quencher is separated from the fluorophore and the primer becomes fluorescent. This allows the monitoring of RPA in real time, merely by using a light source which can excite the fluorophore to fluoresce and using an optical detector to detect any fluorescence from the fluorophore which has separated from the quencher.

The primers for any of the RPA reactions of this disclosure, including the extension blocked primers, may be between 2 to 100 residues in length, such as between 12 to 30

residues in length, 12 to 40 residues in length, 12 to 50 residues in length, or 12 to 60 residues, 30 to 40 residues in length, 40 to 45 residues in length, or 45 to 50 residues in length. In a preferred embodiment, the primers may be between 30 to 100, between 35 to 100, between 40 to 100 or between 45 to 100 in length. In the most preferred embodiment, the primers are between 30 to 60 in length, between 35 to 60, between 40 to 60 or between 45 to 60 in length - these primers may be used in any RPA reactions and are especially preferred for RPA reactions below 30°C degrees, below 15°C degrees or below 20°C. Primers lengths of greater than 30, greater than 35, greater than 40, greater than 45 or greater than 50 bases are preferred for RPA processes performed at or below 30°C. It is understood that in the field of molecular biology, the subunits of a nucleic acid are referred to as "bases" or "residues." For example, DNA and oligonucleotide structures and lengths are referred to in bases (kilobases), basepairs or residues.

Any of the RPA reaction of the invention may be performed between 14°C and 21°C, between 21°C and 25°C, between 25°C and 30°C, between 30°C and 37°C, between 38 °C to 40 °C or between 40°C and 48°C. Applicants have found that RPA reactions are optimal at 25°C in the presence of between 1% to 12% percent PEG. Preferably, the concentration of PEG is between 6 to 9% such as, for example between 7 to 8%. These optimal RPA conditions applies to the RPA reactions disclosed in this application and to all RPA reactions in general.

In a typical RPA reaction of the invention, at least one strand of the target nucleic acid is amplified at least 10^7 folds, at least 10^8 folds or at least 10^9 folds.

For any of the RPA methods of the invention, it is understood that the target nucleic acid may be single stranded. Single stranded nucleic acid may be converted to double stranded nucleic acid by methods known in the art including, for example, the hybridization of random primers followed by elongation by polymerase. Furthermore, the RPA reaction may be performed directly with single stranded target nucleic acid because in a first step, a RPA primer would hybridize to said single stranded target nucleic acid and extension (in the presence of nuclease in the case of the first extension blocked primer) by polymerase would generate a double stranded target nucleic acid for subsequent RPA. Further, a specific primer may be added at the beginning of the RPA reaction to hybridize to the single stranded target nucleic acid and by extension with polymerase already present in the RPA reaction, convert the single stranded target nucleic acid into a double stranded target nucleic acid.

To reduce background and contamination, any of the RPA reactions of the invention may be performed with dUTP in the dNTP mix. We have found, surprisingly, that an RPA may be performed in the presence of dUTP and active uracil glycosylase for a first period before the uracil glycosylase is inactivated. This first period is preferably less than 20 minutes, less than 10 minutes, less than 5 minutes or less than 2 minutes. Furthermore, the uracil glycosylase may be added at any time during the first period. That is, the RPA reaction may be started with dUTP (and other dNTPs) without uracil glycosylase and the uracil glycosylase may be added at any time during the first period.

After the first period, uracil glycosylase inhibitor is added to the RPA reaction and the reaction is allowed to continue for the remainder of the RPA reaction - until a desired degree of amplification is reached. Importantly, the process is performed without temperature based inactivation of the uracil glycosylase. The uracil glycosylase inhibitor in this reaction may be a Bacillus subtilis phages PBS1 uracil glycosylase inhibitor or Bacillus subtilis phages PBS2 uracil glycosylase inhibitor. Where dUTP is used, for any RPA of this disclosure, the dNTP may consist of (1) dTTP, dATP, dUTP, dCTP and dGTP or (2) dATP, dUTP, dCTP and dGTP. In a preferred embodiment, when dUTP is used, the dNTP mixture does not contain dTTP. This method of reducing background, by adding dUTP and uracil glycosylase to a first portion of an RPA reaction has general applicability to any type of RPA. Further, this method may be combined with any of the RPA processes of this disclosure.

Another aspect of the invention relates to a method of performing RPA of a double stranded target nucleic acid molecule comprising a first and a second strand of DNA with an increased signal to noise ratio. In step A, a recombinase agent is contacted with (1) a first extension blocked primer which comprises one or more noncomplementary or modified internal residue which can be a modified internal residue, and (2) a second nucleic acid primer to form a first and a second nucleoprotein primer.

In step B, the first and second nucleoprotein primers are mixed with (contacted to) a nuclease and to the double stranded target nucleic acid such that a first double stranded structure (part of a first D-loop) between the first nucleoprotein primer and said first strand of DNA at a first portion of said first strand is formed. Furthermore, a second double stranded structure (part of a second D loop) between said second nucleoprotein primer and said second strand of DNA at a second portion of said second strand is also formed. The 3' ends of the first extension blocked primer and said second nucleic acid primer are oriented toward each other on the same double stranded target nucleic acid molecule. The nuclease specifically

recognizes and cleaves the one or more noncomplementary or modified internal residue in the first extension blocked primer only after the primer forms a double stranded structure. After cleavage by the nuclease, the first extension blocked primer is cleaved into two primers, a first 5' primer and a first 3' extension blocked primer. Because the blocking group is on the 3' end of the first extension blocked primer, the first 5' primer is not blocked but the first 3' extension blocked primer is blocked and cannot be elongated by polymerase.

In step C, the 3' end of the first 5' primer and second nucleoprotein primer is extended with one or more polymerases and dNTPs (e.g., a mixture of dATP, dTTP, dCTP, and dGTP) to generate an amplified target nucleic acid. The amplified target nucleic acid may be single stranded (for example a displaced strand) or double stranded. Furthermore, single stranded amplified target nucleic acid may hybridize to form double stranded target nucleic acid. Furthermore, the RPA system of this disclosure can amplify both single stranded target nucleic acid (discussed below) or double stranded target nucleic acid so the production of single stranded or double stranded amplified target nucleic acid would not affect the outcome of RPA.

Step B and step C are repeated until a desired degree of amplification is reached. It should be noted that the RPA reaction is self perpetuating as long as the reagents do not run out. The product of one round of amplification (amplified target nucleic acid) serves as the input for subsequent round of RPA. Thus, an RPA reaction may be continued by merely continued incubation of the reaction at a desired temperature. Furthermore, since the RPA reaction disclosed is not temperature sensitive, the reaction may be continued even if there if fluctuation in the temperature. For example, a RPA reaction tube may be performed in a waterbath, on the bench top (room temperature), or even in the pocket of the experimentor (when working in the field, for example). Thus, the RPA reaction may be performed at less than 50°C, less than 40°C, less than 37°C, less than 30°C, less than 25°C, or less than 20°C.

In a preferred embodiment, the first extension blocked primer further comprises one or more detectable labels. Where the detectable label is a fluorophore or a quencher, it may be attached to the extension blocked primer by a fluorophore-dT amidite residue or quencher-dT amidite residue respectively. Other attachments are possible and widely known.

In another preferred embodiment, the extension blocked primer comprises both a fluorophore and a quencher. The fluorophore and quencher may be separated by between 0 to 2 bases, 0 to 5 bases, 0 to 8 bases or 0 to 10 bases. Naturally, it is preferred that the fluorophore and the quencher be sufficiently close to each other such that the combination is

not fluorescent until they are separated. It is preferred that the fluorophore and quencher are separated by a greater distance in the nucleoprotein primer than when the primer is hybridized to the target nucleic acid. This is possible because of the action of the attached proteins (recombinase and/or SSB protein) which tend to stretch out the unhybridized primer.

In another aspect, either fluorophore or the quencher may be attached to the modified internal residue and the fluorophore and quencher can be separated following cleavage of the modified internal residue by the nuclease.

While any fluorophore may function for the methods of the invention, fluorescein, FAM and TAMRA are preferred fluorophores. The preferred quencher is a dark quencher which may be, for example, Dark Quencher 1, Dark Quencher 2, Black Hole Quencher 1 or Black Hole Quencher 2.

Another aspect of the invention is directed to an RPA process of DNA amplification of a single stranded target nucleic acid molecule comprising the steps of (a) hybridizing a first nucleic acid primer to said single stranded target nucleic acid and elongating said primer one or more polymerases and dNTPs to generate a double stranded target nucleic acid molecule comprising a first and a second strand; (b) contacting a recombinase agent with a first extension blocked primer which comprises a noncomplementary internal residue, and a second nucleic acid primer to form a first and a second nucleoprotein primer; (c) contacting the first and second nucleoprotein primers to a nuclease and to said double stranded target nucleic acid thereby forming a first double stranded structure between said first nucleoprotein primer and said first strand of DNA at a first portion of said first strand and a second double stranded structure between said second nucleoprotein primer and said second strand of DNA at a second portion of said second strand such that the 3' ends of said first extension blocked primer and said second nucleic acid primer are oriented toward each other on the same double stranded target nucleic acid molecule, wherein said nuclease specifically cleaves said modified noncomplementary internal residue only after the formation of said first double stranded structure to form a first 5' primer and a first 3' extension blocked primer; (d)extending the 3' end of said first 5' primer and second nucleoprotein primer with one or more polymerases and dNTPs to generate an amplified target nucleic acid molecule; (e)continuing the reaction through repetition of (c) and (d) until a desired degree of amplification is reached. As explained above, the first nucleic acid primer may be the first extension blocked primer, said second nucleic acid primer, first nucleoprotein primer or second nucleoprotein primer. Naturally, if the first primer is the first extension blocked

primer, step (a) should be performed in the presence of the nuclease. Further, it should be noted that any RPA reaction which uses a single stranded nucleic acid target DNA as a starting material will necessarily go through an intermediate stage where the target nucleic acid is double stranded and would be amplified by double stranded amplification.

Another aspect of the invention is directed to a primer for RPA which is an extension blocked primer of between 12 to 100 residues in length and wherein the primer comprises one or more modified internal residues. This primer may be any of the extension blocked primer, including any variants thereof, described anywhere in this application. Briefly, the modified internal residue is selected from the group consisting of a uracil residue, an inosine residue, 8-oxoguanine, thymine glycol, an abasic site mimic and analogs thereof. The abasic site mimic may be a tetrahydrofuran residue or a 5' - O - Dimethoxytrityl-1',2' - Dideoxyribose-3' - [(2-cyanoethyl) - (N,N-diisopropyl)]-phosphoramidite (commonly known as a "D-spacer") and analogs thereof.

The primer is extension blocked and cannot be elongated by polymerase (e.g., Klenow fragment) and dNTP. Methods of blocking a primer from extension are known and are also described in this disclosure. Briefly, the primer may have a blocked 3' residue. The blocked 3' residue may be a blocking moiety. The blocking moiety, which optionally may comprise a detectable label, may be attached to the 2' or 3' site of the 3' most residue of the primer. For example, the blocked 3' residue may be a 2'3'-dideoxy nucleotide.

In another embodiment, the primer comprises one or more detectable labels. The detectable label may be a fluorophore, an enzyme, a quencher, an enzyme inhibitor, a radioactive label, one member of a binding pair and a combination thereof. In a more preferred embodiment, the primer comprises both a fluorophore and a quencher. The quencher may be close to the fluorophore to suppress the fluorescence of the fluorophore. For example, the separation between the fluorophore and the quencher may be 0 to 2 bases, 0 to 5 bases, 0 to 8 bases, 0 to 10 bases, 3 to 5 bases, 6 to 8 bases, and 8 to 10 bases. In a preferred embodiment, the fluorophore and quencher are separated by a greater distance when the extension blocked primer is unhybridized (but attached to recombinase and/or single stranded binding protein) than when the extension blocked primer is hybridized to the target nucleic acid. The fluorophore and quencher may be any fluorophore and quencher known to work together including, but not limited to, the fluorophore and quenchers any of the flurorophores described in this disclosure.

BRIEF DESCRIPTION OF THE DRAWINGS

Figure 1 depicts experimental data showing that lengthening primers accelerate reaction kinetics in the case of primers targeting a *Bacillus subtilis* genomic locus.

Figure 2. depicts experimental results showing only the longer (45-mer) and faster primers successfully amplify DNA to gel detectable levels using ethidium bromide stain at 25°C, 23°C, 20°C, and 17°C.

Figure 3. depicts amplification kinetics at 25°C appear roughly half those at 37°C. This figure also shows that PEG levels influence both rate and specificity (a primer artifact is increased at high PEG concentrations).

Figure 4. shows that primers for the Human ApolipoproteinB locus, ApoB4 and Apo300, demonstrate rapid kinetics when only 33 and 32 residues respectively in length, and reaction kinetics (at 37°C) are not accelerated by elongation.

Figure 5. shows that primers for the Human ApolipoproteinB locus, ApoB4 and Apo300, demonstrate amplification at 25°C regardless of whether the 3' end is elongated.

Figure 6. shows that UNG inhibitor peptide from *Bacillus* phage can be used in combination with *E.coli* UNG for a carry-over contamination system which avoids a need for thermal denaturation of UNG.

Figure 7. depicts experimental data showing (a) A real-time detection probe comprising a FAM fluorophore, (b) a deep dark quencher, (c) an abasic site mimic, and (d) a blocked 3' end, provide excellent characteristics in RPA reactions for monitoring specific product accumulation.

Figure 8. depicts the development of a third probe detection system. Fluorescence data may be best interpreted through a process of normalization and plotting the log of fluorescence.

Figure 9. depicts the use of reversibly blocked primers to gain high signal to noise ratios for sandwich assays. RPA reactions configured with a blocked, splittable, probe active only after splitting by Nfo enzyme can be analyzed directly on lateral flow test strips.

Figure 10. depicts experimental results showing development of a dual-probe amplification/detection system for the hospital superbug MRSA.

Figure 11. depicts real-time probe-based detection of control MSSA DNA sequences.

Figure 12 depicts a schematic of an RPA process.

Figure 13 depicts the use of specific antibodies to immobilize and detect complexes containing two antigenic labels on a flowstrip.

Figure 14 shows polyacrylamide gel electrophoresis of RPA reactions using primers for the human Sry locus.

Figure 15 shows agarose gel electrophoresis of RPA reactions using primers for the human Apolipoprotein B locus.

Figure 16 depicts an investigation of the minimum oligonucleotides size necessary to support RPA

DETAILED DESCRIPTION OF THE INVENTION

In RPA the isothermal amplification of specific DNA fragments is achieved by the binding of opposing oligonucleotide primers to template DNA and their extension by a polymerase (Figure 1A). Unlike PCR, which requires global melting of the target template, RPA employs recombinase-primer complexes to scan double-stranded DNA and facilitate strand exchange at cognate sites. The resulting structures are stabilized by single-stranded DNA binding proteins (SSBs) interacting with the displaced template strand, thus preventing the ejection of the primer by branch migration. Recombinase disassembly leaves the 3'-end of the oligonucleotide accessible to a strand displacing DNA polymerase in this case the large fragment of *B. subtilis* PolI (Bsu) (See, Okazaki et al., 1964), and primer extension ensues. Exponential amplification is accomplished by the cyclic repetition of this process.

In this disclosure, we showed a number of improvements over the basic RPA process. First, we found that with modifications to standard conditions, RPA may be performed efficiently at 25°C or 30°C. These reaction temperatures allows for equipment-free RPA tests with results in under an hour.

Second, we improved the sensitivity and specificity of RPA reactions by using DNA repair enzymes in the RPA reaction. In this study, we employed a wide spectrum of previously identified repair enzymes directly in RPA reactions to see if these enzymes would have an effect on RPA efficiency and fidelity. We hypothesize that primer artifacts arise in RPA principally by errant extension of short-lived hairpin structures formed by the primers, or possibly by forming primer dimers (PCT Application PCT/IB2005/001560 filed April 11, 2005). Although such events are presumably rare, the high concentration of oligonucleotide in a reaction, typically of the order 10^{12}-10^{13} molecules would tend to promote a significant degree of such events when the concentration of target template nucleic acid (i.e., the nucleic acid to be amplified) is low. It should be noted that these side reactions are distinct in nature from those often reported in PCR in which poorly- related sequences are amplified from complex DNA samples due to low fidelity of extension from hybridization products in which only a limited number of 3' residues are homologous to parts of the sample DNA. In RPA we believe that the primary recombinase-mediated pairing requires significant homology over significant regions, and rather that single-stranded DNA's are the species mainly sensitive to artifacts through snapback events occurring at the relatively low temperatures employed. Because of this distinction, methods for reducing primer artifacts in PCR do not necessarily work in RPA reaction. This distinction is important to comprehending the approach and

mechanism described below for decreasing the background noise generated in the system even in the absence of any target nucleic acids, and the way in which this increases sensitivity by decreasing the competitive primer noise.

We disclose herein the use of primers deliberately modified with a 3'-blocking group (with a biotin, ddC residue, or otherwise), and additionally containing a roughly centrally positioned modified (or absent) base. The internally positioned modification became a nuclease target for a repair endonuclease enzyme, which could split the primer to generate two separate primers only if first paired to a target to generate a stable duplex, and then secondarily processed by the enzyme. If one of the new daughter primers (i.e. the most relatively 5' positioned) possesses, or can subsequently be processed to possess, a free extendable 3' hydroxyl group, then it could subsequently function as a polymerase substrate. In contrast the daughter oligonucleotide positioned relatively 3' would retain the original blocking modification and be unable to function as a polymerase substrate. A dependence on splitting the oligonucleotide to form two duplex hybrids separated by a nick or single-nucleotide gap adds noise reduction to the RPA system as there is little or no opportunity for the un-split primer to be erroneously extended in transient fold-back structures due to the presence of the 3' blocking group. We demonstrate the utility of this approach to reduce primer noise here by showing that trace DNA samples can be detected and discriminated from water merely by assessing whether two labeled DNA primers become physically linked. The possibility of such simple assays presents RPA as a powerful tool in the development of cheap, disposable, equipment-free DNA tests.

Finally we have adapted the above duplex-specific nuclease system to the development of proprietary real-time fluorescent probes. We anticipated that the design of effective fluorescent probes would be quite distinct in the RPA system in comparison to other described systems, such as in the PCR method. Why is this? We identified two key areas of difference. First, the organization of the functional groups on the probe would likely be necessarily different due to the extreme difference between RPA reaction environments and those of other amplification systems. Earlier work demonstrated that the RPA reaction environment was fundamentally and critically distinct from that encountered in other nucleic acid amplification reactions. Saturating quantities of single-stranded DNA binding protein and recombinase protein ensures that oligonucleotides with non-modified backbones do not adopt a random coil structure. DNA's are relatively 'stretched out' and rigid as these proteins imbue the nucleoprotein filament with a filament length roughly 1.5 times that of B-form

DNA (Yang et al., 2001; Scheerhagen et al., 1985; Kuil ME et al., 1990). Consequently the supposition that probes covalently linked to fluorophores and quenchers distant in the primary sequence will still quench due to frequent random approach does not hold true. The second key area in which RPA probes were anticipated to be quite distinct form those in other described systems relates to the enzymes employed in probes processing. We discovered experimentally that described approaches using the 5' exonuclease domain of Pol I class enzymes appeared incompatible with RPA (so-called 'Taqman' method), likely due to FLAP endonuclease activity of these enzymes (Kaiser et al., 1999). We further anticipated that other systems such as molecular beacons or scorpion probes were similarly unlikely to be practical (due to the instability of short duplex anchors in RPA conditions). Instead, we here show that it is possible to configure excellent real-time RPA probes by placing fluorophore and quencher moieties close to one another separated by a modified base that leads to backbone splitting only in a duplex context. This approach promises to add tremendous value to the RPA process as it brings the real-time quantitative detection and multiplexing specifications into alignment with the current state-of-the-art using the other methods. Specifically it provides an approach to assess absolute numbers of target nucleic acid molecules in a sample, to increase specificity and sensitivity to allow single molecule detection, and also to permit multiplex analysis of several targets. All of these properties can be attained using this method without a need for gel electrophoresis, or other approaches requiring experimental intervention, but rather reactions can be monitored continuously and automatically by dedicated equipment. To illustrate the power of combining the RPA process with these highly fidelitous detection approaches we have developed an ultra-sensitive, internally-controlled, test for the hospital pathogen MRSA, a difficult target due to the complex and diverse nature of pathogenic strains, and a need for multiplexing.

Each aspect of the invention is described in more detail below:

Low temperature RPA

RPA reactions operate optimally at about 37°C, reflecting the temperature optimum of the enzymes involved in an RPA reaction. While 37°C is easily achieved in the laboratory, an RPA reaction that can function efficiently at 30°C or 25°C would increase the utility of RPA and allow real time amplification under field conditions where a 37°C incubate in not available.

To determine if primer length has an effect on RPA efficiency, RPA reactions were performed at 37°C with primer pairs of different lengths (Figure 1). The results of the

experiments, as shown in Figure 1, shows that primer 'rates' can be enhanced by lengthening primers. Panel A of Figure 1 shows the primer organization at the B. Subtilis locus targeted by BsA1 and BsB3 primers for RPA amplification. The primers BsA1 and BsB3 (30 and 31 residues respectively), or derivatives containing extensions which retain appropriate homolog with the target which were used in the RPA reactions. Panel B shows the results of amplification kinetics monitored in a BIOTEK Flx-800 microplate reader with heated stage set to 38°C. SYBR-green was employed to assess DNA accumulation. Precise reaction conditions and component concentrations are as follows: 10 copies/μl; 10 mM Mg acetate; 50 mM Tris pH 7.9; 100 μM dNTPs; 600 ng/μl gp32; 120 ng/μl uvsX; 30 ng/μl uvsY; 300 nM oligos; 5% Carbowax 20M; 1:50,000 SYBR green; 100 mM Pot. acetate; 20 mM Phosphocreatine; 100 ng/ml CK (creatin kinase); 3 mM ATP.

It is understood that the primers for any of the methods of the invention may be made from DNA, RNA, PNA, LNA, morpholino backbone nucleic acid, phosphorothiorate backbone nucleic acid and a combination thereof. Combinations thereof in this case refer to a single nucleic acid molecule which may contain one or more of one base connected to one of more of another base. Preferred concentration of these molecules may be in the range of between 25 nM to 1000 nM. In one preferred embodiment, the primers may contain a non-phosphate linkage between the two bases at its 3' end and is resistant to 3' to 5' nuclease activity.

Our results show that there was a gradual increase in kinetic rate as the primers were lengthened. In fact lengthening the primers from 30/31-mers to 45-mers cut the amplification time to threshold detection by about 10 minutes, from roughly 35 minutes to 25 minutes under the conditions used here (10mM magnesium, 5% carbowax 20M). Based on the results of this experiment, we conclude that primers with slow kinetics may be enhanced by increasing primer length.

We also investigated whether primer length has an effect on RPA performed in lower temperatures. RPA may not work at a lower temperature for at least two reasons. First, there can be a sudden and dramatic cessation of RPA reaction function below a certain temperature if, for example, one of the components of the reaction cease to function below a certain temperature. For example, the carbowax may go through a phase transition at a lower temperature and cease to function in the desired fashion. Second, the reaction rate may simply slow progressively so that doubling times lengthen, a reflection of slower enzyme catalysis and diffusion. In the second case, the primer 'rate' could be very important because

the reaction would possibly be 'up-against-the-clock' with regard to exhaustion of reaction components such as ATP.

To test our hypothesis, we attempted to amplify the same fragments as in Figure 1 but at 25oC. The results, shown in Figure 2, indicate that primers with fast kinetics can amplify DNA at typical ambient (room) temperatures. The primers used in figure 1 were used to amplify a specific fragment from the *B.subtilis* genome. Figure 2A shows the schematic arrangement of primers. Figure 2B shows that only 45-mers amplify to detectable levels at 25°C. Conditions used were: 50mM Tris pH 8.4, 100mM Potassium acetate, 10mM Magnesium acetate, 2mM DTT, 7.5% PEG compound (Carbowax-20M), 3mM ATP, 25mM Phosphocreatine, 100ng/μl creatine kinase, 700ng/μl gp32, 160ng/μl uvsX, 40ng/μl uvsY, 200 μM dNTPs, 300nM each oligonucleotide. Reaction time, 90 minutes. Start copy density 2 copies/μl, reaction volume 50μl. Figure 2C shows that only 45-mers amplify DNA at 23°C, and amplification to detectable levels can also occur at 20°C and 17°C when the 45-mer is used although progressively less amplification product was recovered. Conditions used: 50mM Tris pH 8.4, 100mM Potassium acetate, 14mM Magnesium acetate, 2mM DTT, 7.5% PEG compound (Carbowax-20M), 3mM ATP, 50mM Phosphocreatine, 100ng/μl creatine kinase, 650ng/μl gp32, 125ng/μl uvsX, 40ng/μl uvsY, 200 μM dNTPs, 300nM each oligonucleotide. Reaction time, 120 minutes. Start copy density 1 copy/μl, reaction volume 20μl.

As seen in Figure 2, specific amplification of about 10^{10} fold observed even at temperatures at low as 17°C. The time to detection was within 2 hours. In the experiments performed at 23°C or below only 20 copies of genomic DNA were added, and although some trace carry-over contamination had been in evidence from water controls (not shown), the attainment of visible product when using ethidium bromide stain (estimated 20ngs at 17°C) suggests an amplification level of around 10^9-fold, or 30 cycles. Importantly high levels of 'noise' are not apparent, although we did observe one additional fast-migrating extra band of unidentified nature (quite possibly classical primer dimer, or single-stranded DNA related to the product).

The kinetic behavior of the 45-mer primers at 25°C, under different concentrations of PEG, is shown in Figure 3. In Figure 3, the 45-mer primers used in figures 1 and 2 were used to amplify a fragment of the *B. subtilis* genome at 25°C. Figure 3A shows the arrangement of the primer pair used. Figure 3B shows agarose gel electrophoresis and ethidium bromide

staining" of samples at reaction endpoint. The expected band (*) is accompanied by an additional band at higher PEG concentrations (#). Figure 3C shows the kinetics of the amplification reaction monitored using SYBR-green. Conditions used was as follows: 50mM Tris pH 8.4, 100mM Potassium acetate, 10mM Magnesium acetate, 2mM DTT, PEG compound (Carbowax-20M) as indicated, 3mM ATP, 25mM Phosphocreatine, 100ng/μl creatine kinase, 650ng/μl gp32, 160ng/μl uvsX, 40ng/μl uvsY, 200 μM dNTPs, 300nM each oligonucleotide, SYBR-green 1:50,000 from stock. Reaction time, 120 minutes. Start copy density 10 copy/μl, reaction volume 50μl.

The lack of a signal in the 4% lane is possibly due to experimental error. The results show that higher PEG concentrations can accelerate kinetics up to a point, and then some inhibition in rate and overall reaction behavior/outcome is observed. In this case 7% or 8% PEG were optimal for maximizing the amount of amplified nucleic acids of the correct length. When the PEG concentrations are higher, there is progressive domination of the faster-migrating anomalous band. In the presence of 8% PEG detection was observed by about 37 minutes at 25°C, which corresponds to a doubling time of around 1 minute 25 seconds. At 5% PEG detection was made at about 54 minutes (corresponding to a 2 minutes doubling time). This reaction at 25°C is about half as fastas the experiment shown in Figure 1 (detection time of 27 minutes and doubling time of 1 minute. Based on this, we estimate RPA reaction rates halve with roughly every 10°C drop in temperature. Further, due to limited pools of reagents such as ATP, detectable product formation may be limited regardless of incubation time depending on the temperature, activity of the primers, and product length. Our results suggest that effective low temperature RPA would be improved with primers that show fast kinetics, and which are not rate limiting in the reaction.

The experiment of Figure 3 was repeated using primers targeting the human Apolipoprotein B gene and the results are shown in Figure 4. Figure 4A shows the arrangement of primers targeting the human Apolipoprotein B locus. Three primer pairs were used as shown, and overlapping primers shared a common 5' extremity but different 3' ends. **(B)** Kinetics of amplification at 38°C. Reactions with the indicated primer pairs were monitored in real-time using SYBR-green dye. Start target copy numbers were either 1 copy/μl or 100 copies/μl of human DNA. Reaction conditions were as follows: 50mM Tris pH 7.9, 100mM Potassium acetate, 10mM Magnesium acetate, 2mM DTT, 5% PEG compound (Carbowax-20M), 3mM ATP, 25mM Phosphocreatine, 100ng/μl creatine kinase,

600ng/µl gp32, 120ng/µl uvsX, 30ng/µl uvsY, 100 µM dNTPs, 300nM each oligonucleotide, SYBR-green 1:50,000 from stock. Reaction time, 60 minutes. Reaction volume 50µl.

Primers for the Human Apolipoprotein B locus show rapid kinetics without primer elongation. In this case kinetic studies using SYBR-green revealed that no rate increase was found with longer RPA primers. It appears that the ApoB4 and Apo300 primers used here, even when short, possess high rate behavior to the extent that they are not the rate limiting factor in the reaction. Presumably, in this reaction, polymerase rate is now the main rate-limiting part of the reaction and more active (longer) primers cannot achieve an overall speed benefit. Consistent with our hypothesis, we find that all of the Apolipoprotein B primers generate the expected product at 25°C (Figure 5). Figure 5A is the same as Figure 4A in that it shows the arrangement of the primers used. Figure 5B shows gel electrophoresis of RPA reactions performed at 25°C using the indicated primer pairs. Copy numbers of zero or 10 copies/µl were tested in each case. Conditions used were as in figure 4 with the exception of the omission of SYBR-green. In this case, no artifact band is seen - supporting the idea that RPA reactions do not significantly suffer from 'noise' at reduced temperatures.

Contamination control using UNG inhibitor from bacteriophage PBS2

RPA reactions are compatible with the use of dUTP as a method to control carry-over contamination. One caveat with the earlier experimental data is that in order to initiate the reaction the uracil glycosylase enzyme had to be heat inactivated. This poses two incompatibility issues with RPA. First, heat inactivation would also inactivate complete RPA reactions because RPA reagents are not heat stable. Second, heat inactivation is inconsistent with one goal of RPA - the avoidance of thermal cycling.

Because of the reasons above, we set to investigate another technical route to implement contamination control. It is known that the *Bacillus subtilis* phages PBS1 (See, Savva and Pearl, 1995) and PBS2 (See, Wang,Z. and Mosbaugh,D.W. (1989)) possess a specific small peptide inhibitor of *E.coli* and *B. subtilis* uracil-DNA glycosylase (Wang and Mosbaugh, 1988). They require a highly effective system as their own DNA is synthesized using dUTP rather than dTTP. We cloned the PBS2 DNA sequencing encoding the inhibitor peptide and expressed it in *E.coli* with a C-terminal hexahistidine tag. We also cloned the *E.coli* uracil glycosylase gene and expressed it with a C-terminal hexahistidine. We used these protein preparations to test whether a carry-over contamination system could be

employed with them. Figure6 shows an example of experiments performed which validate that such an approach. In Figure 6, the start target copy numbers of the template were 800 copies of human DNA where used. Reaction conditions were as follows: 50mM Tris pH 8.4, 100mM Potassium acetate, 10mM Magnesium acetate, 2mM DTT, 5% PEG compound (Carbowax-20M), 3mM ATP, 25mM Phosphocreatine, 100ng/μl creatine kinase, 600ng/μl gp32, 125ng/μl uvsX, 30ng/μl uvsY, 100 μM dNTPs, 300nM each oligonucleotide (SRY8 and SRY9 primers). Reaction time, 75 minutes. Reaction volume 50μl. Where used *E.coli* UNG was used at 150ng/μl, and UNG inhibitor was used at 140ng/μl. Contamination was genuine carry-over contamination present for this amplicon in the laboratory liquid-handling equipment. Reactions were established with all amplification components apart from the polymerase. Reactions 1-4 carried genomic template DNA, reactions 5 and 6 contained only contaminating material. The samples were treated for 5 minutes with UNG in samples 2, 3, 4, and 6. In samples 2, 4, and 6 UNG inhibitor was added after 5 minutes. In all cases after the 5 minute incubation period, with or without UNG and with or without subsequent addition of UNG inhibitor, polymerase was added to initiate DNA synthesis. In this experiment we show the following: (1) that *E.coli* UNG will inhibit RPA reactions containing dUTP substrate, (2) that co-inclusion of the inhibitor peptide overcomes this inhibition, (3) that dUTP-containing contaminants can be suppressed from generating amplicons if first treated with *E.coli* UNG and then with the inhibitor, but that bona fide templates are still effective. Under the conditions used we have seen some evidence of some decrease in robustness/product level when UNG was present in the reaction. We anticipate however that the system may be configured more optimally.

Fluorescent real-time probes for RPA reactions

Many possible applications of the RPA process in detecting DNA (or RNA) sequences would benefit from being applied in a real-time format. RPA has already been shown to be effective when combined with minor groove binding dyes such as SYBR-green (PCT Application PCT/IB2005/001560 filed April 11, 2005). However there may be potential limitations of using such general indicators of DNA accumulation to assess reaction behavior. First, there is no capacity for multiplexing amplification reactions as the dyes cannot discriminate between the various products formed. In many clinical tests, for example, there would be a need to include an internal amplification control to exclude false negatives. Second, RPA reactions are similar to most other DNA amplification processes insofar as even when no target is present in a sample, some DNA synthesis will eventually

ensue. Consequently may be difficult or impossible to discriminate between the presences of a few copies of target nucleic acid or no copies of a nucleic acid based on current methods of florescent detection.

In response to these issues we have developed a proprietary fluorescence-based probe system to monitor RPA reactions. We investigated using the 5'-3' nuclease associated with the polymerases of the *E.coli* Pol I class. This nuclease is used in a fluorescent probe methodology for PCR known as the 5'nuclease, or 'Taqman', assay. We found that both *Bacillus subtilis* Pol I retaining the 5'-3' nuclease domain and the *E.coli* PolI enzyme would not support RPA reactions. On reflection we believe this arises because these nucleases are structural/functional homologs of the FEN1 FLAP endonuclease family and most likely are structure-specific endonucleases (Kaiser et al.). We suppose these enzymes progressively digest the displaced strand during the strand-displacement synthesis thus inhibiting DNA amplification.

We focused our attentions particularly on the *E.coli* glycosylase enzymes and AP endonucleases involved in DNA repair known as fpg, Nth, Nfo, and more recently *E.coli* exonuclease III. Importantly these enzymes will only remove damaged bases and/or nick DNA backbones at positions in which base modifications have occurred and, critically, in the context of duplex DNA. All of these enzymes are able to cleave such appropriate duplex DNA molecules with high specificity in the RPA environment (see application). Test probes were utilized that contained a modified base within the body of the oligonucleotide (8-oxoguanine, thymine glycol, or abasic site mimic respectively) and an additional distinct elongation blocking group on the 3' end (provided by a 3'-dR-biotin). Despite obvious promise for all of these enzymes, and potentially other repair/processing enzymes, we focused on the behavior of the *E.coli* Nfo and exonuclease III enzymes for the following reasons. First, we observed when testing fpg, Nth, and Nfo proteins that the degree of successful probe processing was highest for the probe containing a tetrahydrofuran residue (THF - an abasic site mimic), and processed by Nfo. Second, because Nfo, and the functionally similar *E.coli* exonuclease III, split the oligonucleotide into two smaller oligonucleotides separated by a single nucleotide gap, in which the new 3' end that is formed can be elongated by a strand displacing polymerase that can initiate at nicks. This property endows the THF/Nfo or THF/exonuclease III processing system with a wealth of application opportunities that extend beyond application to fluorescent probe processing. (Note that other abasic site mimics, or true abasic sites might also be employed).

" "" A previous report has also illustrated a potential use of employing an abasic, or other blocking residue, in the context of an amplification process, with the preferred intention to remove the residue in the context of PCR or LCR reactions using a thermostable nuclease (US patent 5,792,607, referred to herein as the '607 patent). However the approach we used is distinct from that of the '607 patent. In the '607 patent, an abasic site is described as one member of a broader selection of modifying groups, to be positioned preferentially at the 3' end of the intended amplification oligonucleotide, and designed to serve as a reversible 3' sugar modifying group by effectively preventing substrate recognition or catalysis by the polymerase. The intention is to decrease the propensity of the amplification system to amplify unintended targets in sample DNA because of the tendency of PCR and LCR techniques to form, albeit at reduced frequency, hybrids with sequences sharing limited homology to the 3'-region of oligonucleotide primers. Furthermore it is intended, critically, in the '607 patent that this modification preventing substrate recognition be specifically corrected in a target-dependent fashion. Such an activity might be performed by the activity of an agent such as endonuclease IV which can 'polish' groups from a 3' sugar residue. However, quite distinctly, in the process described herein the THF residue does not serve as an elongation-blocking modification agent to the 3' sugar that prevents the initial oligonucleotide/template hybrid being recognized as a bona fide substrate. Indeed the THF residue, instead of being located at the very 3' end of an oligonucleotide, is positioned within the body of the oligonucleotide, away from the substrate target of the polymerase (i.e. the 3' end region of the hybridized primer on the template DNA). In this disclosure the principal motivation is to prevent noise arising from primer fold-back artifacts. Thus, instead, herein the processing of the THF residue by an endonuclease activity leads to incision of the oligonucleotide backbone in the context of a bona fide duplex in a distinct event from 'correction' of the modification that prevents polymerase substrate recognition. We also describe herein 3' terminal elongation-blocking modifications, however these are not the 'corrected' modification in this case, and are not necessarily removed from 3'-terminal nucleotides as in the '607 patent. Instead, in the case described here we would employ two separable entitities, a non-corrected 3'-blocking group, and a centrally located abasic-like residue which can be incised by an AP endonuclease to generate a nicked structure and two independent daughter annealed primers, only one of whom is a polymerase substrate.

Figure 7 shows the results of an experiment in which a fluorescent sensing probe has been employed to assay for the accumulation of a specific amplicon in an RPA reaction.

Figure 7A shows a schematic structure of the probe. The probe has internal base-labeled fluorophore and quencher (fluorescein and deep dark quencher II) which were incorporated during synthesis by using commercially available (Glen Research, Sterling, Virginia, USA) fluorescein-dT or DDQ2-dT amidites.

A THF residues was inserted at a nucleotide position between these modified bases. The probe was blocked by the presence of a 3'-dR-biotin group. Figure 7B shows the probe sequence which is:

5'-catgattggatgaataagctgcagc (dTfluoro) g (THF) t (dT-DDQ1)aaaggaaactta-dRbiotin-3' (SEQ ID NO:8)

The probe is homologous to part of the *Bacillus subtilis* SpoOB locus contained within an amplicon generated by primers J1 and K2. The fluorophore and quencher were designed to be on T residues in the sequence so that they could be incorporated directly on commercially available amidites. Figure 7C shows the amplification and probe cleavage kinetics as monitored by fluorescence increase. Amplification reactions were established with varying concentrations of target *Bacillus subtilis* genomic DNA. Reactions were established on ice and then incubated in a BIOTEK Flx800 microplate reader with stage set at 38°C. Amplification conditions are as follows: Start target copy numbers were as indicated. Reaction conditions: 50mM Tris pH 7.9, 100mM Potassium acetate, 12mM Magnesium acetate, 2mM DTT, 5% PEG compound (Carbowax-20M), 3mM ATP, 25mM Phosphocreatine, 100ng/μl creatine kinase, 900ng/μl gp32, 120ng/μl uvsX, 30ng/μl uvsY, 180ng/μl Nfo, 100 μM dNTPs, 450nM of K2 primer, 150nM J1 primer, 100nM probe. Reaction time, 60 minutes. Reaction volume 20μl.

The sensing probe was designed to possess a fluorophore and quencher separated by (a) less than 10 bases (to ensure efficient quenching) and (b) a cleavable site (THF residue). In this case the primary amplicon was generated using the primers J1 and K2 to amplify a fragment from the *Bacillus subtilis* SpoOB locus. RPA reactions were modified from our usual conditions in the following manner. First the probe was included, whose overall structure and sequence is shown in the lower part of the figure. Second the amplification primers were biased in concentration so that there was a relative excess of the amplification primer opposing the probe in order that there might be a steady-state excess of complementary sequences to the probe. Finally the Nfo enzyme was included in the reaction. Reactions were performed in 20 microliter volumes in a standard 384-well plate and

fluorescence monitored using excitation/detection filters of 485/525 in a BIO-TEK Flx800 plate reader. We observed that there was a template-dependent increase in fluorescence. The time at which accumulation begins was dependent on the copy number, as was the level of total fluorescence at the end of the period of reaction monitoring at one hour.

In figure 8 this experiment was repeated. Figure 8A shows the raw fluorescence data while Figure 8B shows normalized fluorescent signals. The fluorescence signal present in the water control at any given time was subtracted from all other sample fluorescence signals. All samples were normalized to one another by adjusting them to a common baseline based on the period prior to measurable fluorescence rise. In Figure 8C, the log of the normalized fluorescence data was plotted and in Figure 8D the time of threshold crossing of the fluorescence signal (set to about 2.6) was plotted against start copy number.

In this case we have shown the result of normalizing the samples against the signal in the water control, and then the results of plotting the logarithm of the normalized fluorescence signal. We set a fluorescence signal of 2.5 or above as constituting a positive signal. Note that it is easy to distinguish the low copy samples from water in contrast to the situation usually observed when using SYBR-green. The slight fluorescence increase in the water sample is almost certainly due to slight carry-over contamination associated with this particular amplicon which has been handled widely in the laboratory.

With respect to the quenchers of this disclosure, it is understood that a quencher need not be a fluorophore. A non-fluorescent chromophore can be used that overlaps with the donor's emission (a dark quencher). In such a case, the transferred energy is dissipated as heat.

High efficiency dark quenchers, such as Dark Quencher 1, Dark Quencher 2 and Black Hole Quencher1 and Black Hole Quencher 2 are known and commercially available (Biosearch Technologies, Inc., Novato, Calif.). As is known in the art, the high quenching efficiency and lack of native fluorescence of the dark quencher allows attachment of a fluorophore and a quencher on one oligonucleotide and ensures that such an oligonucleotide does not fluoresce when it is in solution.

Suitable fluorophores and quenchers for use with the polynucleotides of the present invention can be readily determined by one skilled in the art (see also, Tgayi et al., Nature Biotechnol. 16:49-53 (1998); Marras et al., Genet. Anal.: Biomolec. Eng. 14:151-156 (1999)). Many fluorophores and quenchers are available commercially, for example from

Molecular Probes (Eugene, Oreg.) or Biosearch Technologies, Inc. (Novato, Calif.). Examples of fluorophores that can be used in the present invention include, but are not limited to, fluorescein and fluorescein derivatives such as FAM, VIC, and JOE, 5-(2'-aminoethyl)aminonaphthalene-1-sulphonic acid (EDANS), coumarin and coumarin derivatives, Lucifer yellow, NED, Texas red, tetramethylrhodamine, tetrachloro-6-carboxyfluoroscein, 5 carboxyrhodamine, cyanine dyes and the like. Quenchers include, but are not limited to, DABSYL, 4'-(4-dimethylaminophenylazo)benzoic acid (DABCYL), 4-dimethylaminophenylazophenyl-4'-maleimide (DABMI), tetramethylrhodamine, carboxytetramethylrhodamine (TAMRA), Black Hole Quencher, Dark Quencher 1, and Dark Quencher 2. Methods of coupling fluorophores and quenchers to nucleic acids are well-known in the art.

We have successfully implemented a fluorescent probe system in the RPA reaction environment and established the general structure of probes. With this knowledge it should be easy to develop probes to detect any amplicon, and by judicious selection of alternate fluorophores, to multiplex more than one amplification at once. To demonstrate this we have developed a multiplex test for the antibiotic-resistant *S.aureus* pathogen known in the United Kingdom as methicillin-resistant *Staphylococcus aureus*, or MRSA for short.

The Detection of methicillin-resistant *Staphylococcus aureus*

MRSA comprises a collection of *Staphylococcus aureus* strains which have developed antibiotic resistance by integration of a resistance cassette, the mecA cassette, at a specific location in the *S. aureus* genome. While the same general genomic integration site is always used, the precise integration site junctions and orientation of the cassettes can vary. Despite this variation, independent isolates can be segregated into a limited number of general groups with representative integration structures. In addition to this complexity, further difficulties arise due to the existence of base polymorphisms between strains which can compromise the effectiveness of amplification primers and probes. The MRSA pathogen thus represents a complex target because in order to capture over 90% of the strains commonly found in clinical specimens in a single test it is necessary to accommodate detection of three structurally distinct variations of the mecA resistance cassette integration locus, and account for some common polymorphisms. Additionally, it is necessary that the amplicon spans one arm of the integration cassette to ensure that any mecA sequences amplified are in the context of the *S.aureus* genome, and were not present in an unrelated bacterium.

In order to configure an RPA test for over 90% of common MRSA strains, we developed a primer design strategy which is illustrated in figure 10. Figure 10 depicts the real-time detection of MRSA alleles in a multiplex test environment. Figure 10A is a schematic of the RPA probe principle. Signal generation depends on probe cutting by double-strand specific Nfo. Figure 10B depicts an arrangement of primers and probes relative to the targets used in 2C-F and 3C. A PCR fragment that fused an unrelated sequence to the target sites sccIII and orfX served as internal control. Figure 10C shows probe signal of RPA reactions using the primer set orfX/sccIII. MRSAIII DNA at 10^4 (black, reactions 1-3), 10^3 (red, 4-6), 100 (yellow, 7-9), 10 (green, 10-12) or 2 copies (purple, 13-17) or water (blue, 18-20) served as template. Figure 10D shows a plot of the onset time of amplification (defined as passing the 2.5 threshold) in reactions 1-12 in 2C against the logarithm of the template copy number reveals a linear relationship. **(E)** A multiplex RPA approach enables detection of different MRSA alleles and an internal control in the same reaction. MRSAI (green), MRSAII (dark blue), MRSAIII DNA (red) at 10 copies or MSSA DNA at 10^4 copies (blue, negative control) or water (yellow, turquoise) served as a template (in triplicate for each template condition). **(F)** Detection of the 50 copies of internal control DNA included in the reactions in 2E. A negative control contained water (turquoise). The RPA reactions were performed as follows: Real-time RPA was performed in a plate-reader (BioTek Flx-800) in the presence of fluorophore/quencher probes. Reactions were performed at 37°C for 90 minutes. Conditions were 50mM Tris (pH 7.9), 100mM Potassium-acetate, 14mM Magnesium-acetate, 2mM DTT, 5.5% Carbowax20M, 200 μM dNTPs, 3mM ATP, 50mM Phosphocreatine, 100ng/μl Creatine-kinase, 20ng/μl Bsu. Concentrations of gp32/uxsX/uvsY (in ng/ul) were 900/120/30. Primers were employed at 265nM sccI/II, 265nM sccIII, 70nM orfX. Reaction volumes were 20μl.

Three probes were employed:

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SATamra1  5'- tgttaattga acaagtgtac agagcatt (T)a(H)ga(q1)
tatgcgtgga g-Biotin-3' (SEQ ID NO:9)
SATamra2  5'-tgttaattga gcaagtgtat agagcatt (T)a(H)ga(q2)
tatgcgtgga g-Biotin-3'  (SEQ ID NO:10)
BSFlc     5'-catgattgga tgaataagct gcagc (F)g(H)t(q3)
aaaggaaact ta-Biotin-3'  (SEQ ID NO:11)
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Here (T) is dT-TAMRA, (F) is dT-Fluorescein, (H) is THF, (q1) is dT-BHQ1, (q2) is dT-BHQ2, (q3) is dT-DDQ1. Probes were employed at 60nM SATamra1 (MRSAIII

experiment) or at 45nM SATamra1, 45nM SATamra2, 60nM BSFlc (multiplex experiment). Nfo was used at 200ng/ul. Excitation/detection was at 485/525nm (SybrGreenI, BSFlc) or 530/575nm (SATamra1/2). Measurements were taken every 30sec or 45sec (multiplex experiment). Fluorescence probe data were normalized against water control and pre-amplification baseline adjusted. The logarithm of the read-out was plotted against reaction time.

Briefly, a single primer was designed to recognize the *S. aureus* genomic DNA outside of the integration cassette region, and is termed orfX. Two further primers specific to the mec cassette were designed, and one of these (scc I/II) can be used to amplify the locus from two of the strain variants, while the second (scc III) amplified the locus from the third variant. Two probes for the amplicons are used, differing in two residues to account for common single nucleotide polymorphisms. Both these MRSA probes use TAMRA as fluorophore. Finally a control amplicon is included in the reaction which comprises a unique segment of an unrelated *B. subtilis* genomic DNA fragment fused to the orfx and sccIII primers, and a third probe may be used to sense this amplicon (BSFlc, and this is the same probe used in the experiments in Figure 7, contains a fluorescein and deep dark quencher I). Fig 10 part A illustrates once again the strategy for developing increased fluorescence in the reaction by processing of probes forming hybrids with amplicons. In Part C detection of one MRSA genomic DNA template is demonstrated over a wide concentration range in a non-multiplexed environment. Part E shows the results of an experiment in which (approximately) 10 copies of each of the three types of MRSA were separately detected using a single reaction mastermix. In part F the signal generated by the control sequence in the fluorescein channel is shown, and we can see that all those samples containing control DNA score positive.

Included in these experiments are control reactions containing relatively high concentrations of (10^4 copies) of non-resistant *S. aureus* DNA. Satisfyingly, these samples do not score positive indicating a strict requirement for both *S. aureus* sequences as well as the mecA cassette. To ensure that this control DNA was functional and that the copy concentration was as indicated, the DNA was used in control reactions employing a combination of the orfx primer and a second *S.aureus* specific primer termed mssa. In this case the same probes may be employed as the probes recognize common sections of the *S.aureus* genome. In Figure 11 we can observe the results of an experiment performed with these non-resistant strain specific primers, and see how the control MSSA DNA is indeed

effective, and shows appropriate response of the quantitative analysis to copy number. Figure 11 depicts the detection of MSSA DNA in a real-time quantitative RPA reaction. Probe signal of RPA reactions using the primer set orfX/mssa and probe SATamra2. Figure 11A depicts measurement of MSSA DNA at 10^4 (black, reactions 1-3), 10^3 (red, 4-6), 100 (yellow, 7-9), 10 (green, 10-12) or 2 copies (purple, 13-17) or MRSAI DNA at 10^4 copies (grey, reactions 18-20) or water (blue, 21-23) served as template. Reaction conditions were 50mM Tris (pH 7.9), 100mM Potassium-acetate, 14mM Magnesium-acetate, 2mM DTT, 200 μM dNTPs, 3mM ATP, 20mM Phosphocreatine, 100ng/μl Creatine-kinase, 5% Carbowax20M, 900ng/μl gp32, 120ng/μl uvsX, 30ng/μl uvsY and 20ng/μl Bsu. Oligonucleotides were employed at 500nM mssa, 100nM orfX and 60nM SATamra2. Whilst the MSSA target is amplified even at very low concentrations, the negative control (MRSAI) does not generate a signal. Figure 11B depicts a plot of the onset time of amplification (defined as passing the 2.5 threshold) in reactions 1-12 against the logarithm of the template copy number reveals a linear relationship.

Detection of trace nucleic acids by association of primers following enzymatic generation of an extendable 3' end

RPA is ideally suited to the development of portable equipment-free, or equipment-light, DNA tests. However such tests would ideally employ cheap, easy-to-use, approaches to determine whether amplification has occurred. Traditionally gel electrophoresis is used to assess whether a product of a defined size has accumulated. Alternatively fluorescent probes may be employed. In either case significant hardware is required to perform the analysis and this prevents the test being used by end-users lacking appropriate equipment.

Other approaches may be used to determine whether or not DNA amplification has occurred. One convenient hardware-free approach is to perform a sandwich assay in which the presence of an amplicon is assessed by interrogating whether two labeled gene-specific primers have become associated in a common DNA duplex. This can be achieved by labeling one amplification primer with a label, such as biotin, and an opposing primer with a second label, such as FAM. A variety of approaches can be employed to determine whether the two labeled primers become associated. For example in a conventional lateral flow strip assay (see for example patent EP0810436A1), two antibodies (or other moiety such as streptavidin that binds with high affinity to one of the oligonucleotide labels) are employed. One

antibody would be immobilized on a flow membrane in a line or spot. The other is coupled to visible particles such as colloidal gold, latex particles, or similar. When the sample, in this case a diluted or undiluted amplification reaction, is applied to a sample pad in which the antibody-coupled visible particles are pre-deposited, the visible particles become stably associated with one of the labeled oligonucleotides. The entire sample then moves by capillary action up the membrane and as it flows the other labeled primer becomes 'caught' on the immobilized antibody. If the labeled primers are not co-associated in a duplex then the antibodies 'caught' on the membrane are not associated with the visible particles associated with the other primer. If, however, they are associated as a consequence of amplification then the visible particles also become trapped on the line or spot, and a visible signal accumulates. Other approaches to assess for association of primers can be configured.

One problem with simple association assays, such as sandwich assays, is the requirement that the primers do not associate unless bona fide amplification of the desired target has occurred. Any undesired association will lead to a false positive signal. However such a clean-cut situation is rarely the case with most amplification methods, particularly when the target is not abundant. For example primer dimers, or other artifacts, tend to accumulate to some extent in the PCR method regardless of optimization. RPA also suffers from the accumulation of primer-related artifacts as detailed earlier, and these are likely to interfere with the direct combination of RPA with such simple read-outs. Indeed this general problem may underpin part of the reason that sandwich assays have not been broadly implemented in currently available high sensitivity/specificity DNA tests. Those commercially available lateral flow systems marketed to assess PCR product accumulation are inconvenient, requiring a final step of hybridizing an additional probe primer to the product after the reaction has been performed in order to avoid aberrant co-association of primers through DNA synthesis (e.g. The Genline Chlamydia Direct test strip from Milenia).

We have configured RPA reactions to permit easy assessment of bona fide target amplification by direct addition to lateral flow strips, or potentially by other similar methods. To attain a clean distinction between positive and negative samples we have employed a labeled primer which is split by the *E.coli* Nfo or exonuclease III enzymes to generate two primers, one of which may be elongated. This is attained by blocking the 3' end of the oligonucleotide, and separately incorporating a THF residue or product of employing a 5' - O - Dimethoxytrityl-1',2' - Dideoxyribose-3' - [(2-cyanoethyl) - (N,N-diisopropyl)]-phosphoramidite during oligonucleotide synthesis, referred to herein as "D-spacer" available

from Glen Research, Sterling, Virginia, USA) within the oligonucleotide to act as a splitting target for the enzyme. The dependence on formation of a stable duplex before the Nfo or exonuclease III enzymes will incise/split the primer ensures that aberrant association of this primer with the other labeled opposing primer does not occur, or is so infrequent as to fall below threshold of detection.

Figure 9 shows data from experiment in which DNA from a methicillin-resistant *S. aureus* strain (EMRSA 16 strain containing the mec2 cassette), or from a non-resistant reference strain (MSSA) has been subjected to amplification in the presence of 3 primers. This experiment shows that a high signal to noise ratio amplification strategy suitable for lateral flow assays or other simple sandwich detection schemes is feasible. Figure 9A shows a schematic of the arrangement of primers. The left-most primer, and the probe, recognize sequences present in the *S. aureus* genome, and similarly present in the *S.aureus* MSSA reference strain as well as the MRSA16 strain which contains a downstream mecII cassette insert. The right-most amplification primer is specific for sequences in the mecII cassette and is not found in the non-resistant *S.aureus* genome. The right-most primer is 5'-labelled with a biotin moiety, while the probe is labeled with a 5'-FAM moiety. The probe is blocked with 3' ddC, and contains an internal THF residue. In Figure 9B, amplification reactions were established with the following conditions: 50mM Tris pH 7.9, 100mM Potassium acetate, 14mM Magnesium acetate, 2mM DTT, 5% PEG compound (Carbowax-20M), 3mM ATP, 25mM Phosphocreatine, 100ng/μl creatine kinase, 600ng/μl gp32, 125ng/μl uvsX, 30ng/μl uvsY, 270ng/μl Nfo, 100 μM dNTPs, 100nM of ORFX45b primer, 100nM sccII-35-2-bio primer, 50nM probe ORFXprobe2. Reaction time, 60 minutes. Reaction volume 30μl. Reaction temperature 37°C. Copy numbers were 1000 copies of MSSA DNA or 1000 copies of MRSA16 DNA, or water. After 60 minutes 1 μl of the reaction was diluted with 5 μl of PBS/3%Tween-20, and applied to the sample pad of a commercial lateral flow test strip from Milenia using 100μl of PBS/3%Tween-20 (Milenia product: Genline hybri-detect MGHD1).

In this case 2 of the primers act as the main amplification primer pair, and a third acts as a probe. The probe contains a 3' blocking group and a separate internal THF residue to act as a splitting target, as well as a FAM label at the 5' end. The probe opposes one of the main amplification primers which is labeled with a biotin residue. Only if a bona fide amplicon accumulates will the probe form stable hybrids that are nicked/split by Nfo, elongated, and thus associate the 2 labeled primers. The results of an experiment are shown in which RPA amplifications established in this way were performed on DNA from the resistant and non-

resistant strains. A small quantity of the reaction (1 μl) was then mixed with 5 μl of lateral flow running buffer (Phosphate buffered saline with 3% Tween-20) and directly applied to a commercial lateral flow strip (Milenia-germany). After about 1-2 minutes the strips were assessed for signal, and a photograph was taken. The test clearly distinguishes positive from negative.

Other processing enzymes might be employed in such approaches. In particular the *E.co*li fpg, Nth, and exonuclease III enzymes, homologs from other phyla, base mismatch repair enzymes such as *E.coli* MutY, MutS and MutM, *E.coli* MUG, Human MUG, Oggl, and the vertebrate Nei-like (Neil) glycosylases. Any combination of the above repair enzymes might also be employed. In particular note that E.coli Nfo (endonuclease IV), and E.coli exonuclease III, possess phosphodiesterase activities and are capable of processing the non-extendable 3' ends of nicked products of the other glycosylase/lyases to extendable 3'-hydroxyl residues.

All patents, patent applications and references, cited anywhere in this disclosure, are incorporated by reference in their entirety.

The invention will now be described further by way of examples. The examples are illustrative of the invention and are not intended to limit it in any way.

EXAMPLE

Example 1 Nucleic Acid Sequences

Proteins and DNA

Coding sequences for uvsx, uvsy, gp32, Bsu and Nfo were amplified from genomic DNA (DSMZ, Germany), fused to hexahistidine-tags (N-terminal for uvsY, Bsu and Nfo, C-terminal for uvsX and gp32) and cloned into suitable expression vectors. Overexpression and purification was done by standard protocols using Nickel-NTA resin (Qiagen). *S. aureus* alleles were EMRSA-3 (SCC*mec* type I; MRSAI), EMRSA-16 (MRSAII), EMRSA-1 (MRSAIII) and wild-type MSSA. See additional sequence information provided below.

Primer sequences

Human locus ApoB (product size experiment SI):

Apo700 tggtaaacgg aagtctggca gggtgattct cg (SEQ ID NO:12)

Apo800 caattgtgtg tgagatgtgg ggaagctgga at (SEQ ID NO:13)

Apo900 gaggtggttc cattccctat gtcagcattt gc (SEQ ID NO:14)

Apo1000 gggtttgaga gttgtgcatt tgcttgaaaa tc (SEQ ID NO:15)

Human loci for STR markers (STR experiment and primer size experiment, SI):

CSF1PO 5' gttgctaacc accctgtgtc tcagttttcc tac (SEQ ID NO:16)

CSF1PO 3' agactcttcc acacaccact ggccatcttc agc (SEQ ID NO:17)

D7S820 5' gaacacttgt catagtttag aacgaactaa cg (SEQ ID NO:18)

D7S820 3' gaattataac gattccacat ttatcctcat tgac (SEQ ID NO:19)

D13S317 5' ttgctggaca tggtatcaca gaagtctggg atg (SEQ ID NO:20)

D13S317 3' ccataggcag cccaaaaaga cagacagaaa ga (SEQ ID NO:21)

D16S539 5' aaacaaaggc agatcccaag ctcttcctct tcc (SEQ ID NO:22)

D16S539 5' ataccattta cgtttgtgtg tgcatctgta agc (SEQ ID NO:23)

D18S51 5' ggtggacatg ttggcttctc tctgttctta ac (SEQ ID NO:24)

D18S51 3' ggtggcacgt gcctgtagtc tcagctactt gc (SEQ ID NO:25)

THO1 5' tacacagggc ttccggtgca ggtcacaggg a (SEQ ID NO:26)

THO1 3' ccttcccagg ctctagcagc agctcatggt gg (SEQ ID NO:27)

TPOX 5' actggcacag aacaggcact tagggaaccc (SEQ ID NO:28)

TPOX 3' ggaggaactg ggaaccacac aggttaatta (SEQ ID NO:29)

Human loci ApoB, D18S51 and Sry (primer size experiment, SI):

APOB500 atggtaaatt ctggtgtgga aaacctggat gg (SEQ ID NO:30)

APO500-28 taaattctgg tgtggaaaac ctggatgg (SEQ ID NO:31)

APO500-25 attctggtgt ggaaaacctg gatgg (SEQ ID NO:32)

APOB300REV ctatccaaga ttgggctaaa cgtatgaaag ca (SEQ ID NO:33)

APOB300REV-28 ccaagattgg gctaaacgta tgaaagca (SEQ ID NO:34)

APOB300REV-25 agattgggct aaacgtatga aagca (SEQ ID NO:35)

D18S51 5'-28 gacatgttgg cttctctctg ttcttaac (SEQ ID NO:36)

D18S51 5'-25 atgttggctt ctctctgttc ttaac (SEQ ID NO:37)

D18S51 3'-28 gcacgtgcct gtagtctcag ctacttgc (SEQ ID NO:38)

D18S51 3'-25 cgtgcctgta gtctcagcta cttgc (SEQ ID NO:39)

SRY3 aaagctgtaa ctctaagtat cagtgtgaaa c (SEQ ID NO:40)

SRY3-28 gctgtaactc taagtatcag tgtgaaac (SEQ ID NO:41)

SRY3-25 gtaactctaa gtatcagtgt gaaac (SEQ ID NO:42)

SRY4 gttgtccagt tgcacttcgc tgcagagtac c (SEQ ID NO:43)

SRY4-28 gtccagttgc acttcgctgc agagtacc (SEQ ID NO:44)

SRY4-25 cagttgcact tcgctgcaga gtacc (SEQ ID NO:45)

DNAs used in this disclosure

BsA1 ttgggcactt ggatatgatg gaactggcac (SEQ ID NO:46)

BsA1-36 ttgggcactt ggatatgatg gaactggcac ggttgt (SEQ ID NO:47)

BsA1-40 ttgggcactt ggatatgatg gaactggcac ggttgttgcg (SEQ ID NO:48)

BsA1-45 ttgggcactt ggatatgatg gaactggcac ggttgttgcg tccat (SEQ ID NO:49)

BsB3 ccatcttcag agaacgcttt aacagcaatc c (SEQ ID NO:50)

BsB3-36 cgccatcttc agagaacgct ttaacagcaa tccatt (SEQ ID NO:51)

BsB3-40 cgccatcttc agagaacgct ttaacagcaa tccattttgc (SEQ ID NO:52)

BsB3-45 cgccatcttc agagaacgct ttaacagcaa tccattttgc gccag (SEQ ID NO:53)

ApoB4 cagtgtatct ggaaagccta caggacacca aaa (SEQ ID NO:54)

ApoB4-40 cagtgtatct ggaaagccta caggacacca aaataacctt (SEQ ID NO:55)

ApoB4-45 cagtgtatct ggaaagccta caggacacca aaataacctt aatca (SEQ ID NO:56)

Apo300 tgctttcata cgtttagccc aatcttggat ag (SEQ ID NO:57)

Apo300-40 tgctttcata cgtttagccc aatcttggat agaatattgc (SEQ ID NO:58)

Apo300-45 tgctttcata cgtttagccc aatcttggat agaatattgc tctgc (SEQ ID NO:59)

SRY8 ccagctgtgc aagagaatat tcccgctctc cg (SEQ ID NO:60)

SRY9 cctgttgtcc agttgcactt cgctgcagag t (SEQ ID NO:61)

J1 acggcattaa caaacgaact gattcatctg cttgg (SEQ ID NO:62)

K2 ccttaatttc tccgagaact tcatattcaa gcgtc (SEQ ID NO:63)

NfoI probe 5'-catgattgga tgaataagct gcagc-[dTfluorescein]-g-[tetrahydrofuranyl]-t-[dT-DDQ1]-aaaggaaact ta-dRbiotin-3' (SEQ ID NO:64)

ORFX45-b ccaagaattg aaccaacgca tgacccaagg gcaaagcgac tttgt (SEQ ID NO:65)

ORFXprobe2 5'-(FAM)-CCACATCAAATGATGCGGGTTGTGTTAAT-[d-SPACER]-GAACAAGTGTACAGAG-3'ddC (block) (SEQ ID NO:66)

SATamra1 5'-tgttaattga acaagtgtac agagcatt-[dT tamra] a(THF)ga(BHQ1)tatgcgtgga g-Biotin-3' (SEQ ID NO:67).

SATamra2 5'-tgttaattga gcaagtgtat agagcatt(dT tamra])a(THF)ga(BHQ2)tatgcgtgga g-Biotin-3' (SEQ ID NO:68)

BSFlc 5'-catgattgga tgaataagct gcagc (F)g(H)t(q3) aaaggaaact ta-Biotin-3' (SEQ ID NO:69)

Sequence of MSSA and MRSA alleles and primers used here:

Primer target sites are bold/underlined, probe binding site is in bold/italic.

MRSA/MSSA primers (*S. aureus* experiment):

SCCI/II ctcaaagcta gaactttgct tcactataag tattc (SEQ ID NO:70)

SCCIII ccaatatttc atatatgtaa ttcctccaca tctca (SEQ ID NO:71)

ORFX cccaagggca aagcgacttt gtattcgtca ttggcggatc aaacg (SEQ ID NO:72)

MSSA ccaatttgat agggcctaat ttcaactgtt agcta (SEQ ID NO:73)

sccII-35-2-bio 5'-bio-ctatgtcaaa aatcatgaac ctcattactt atgat (SEQ ID NO:74)

MSSA DNA sequence:

ttttagatat aaa**ccaattt gatagggcct aatttcaact gttagcta**ct acttaagtta tatgcgcaat tatcgtgata tatcttatat attgaatgaa cgtggattta atgtccacca tttaacaccc tccaaattat tatctcctca tacagaattt tttagtttta cttatgatac gcct***ctccac gcataatctt aaatgctcta tacacttgct caattaaca***c aacccgcatc atttgatgtg ggaatgtcat tttgctgaat gatagtgcgt agttactgcg ttgtaagacg tccttgtgca ggc**cgtttga tccgccaatg acgaatacaa agtcgctttg cccttggg**tc atgcg (SEQ ID NO:75)

MRSAI DNA sequence:

tttagttgcagaaagaatttt**ctcaaagctagaactttgcttcactataagtattc**agtata aagaatatttcgctattatttacttgaaatgaaagactgcggaggctaactatgtcaaaaat catgaacctcattacttatgataagctt***ctccacgcataatcttaaatgctctatacacttg ctcaattaaca***caacccgcatcatttgatgtgggaatgtcattttgctgaatgatagtgcgt agttactgcgttgtaagacgtccttgtgcaggc**cgtttgatccgccaatgacgaatacaaag tcgctttgcccttggg**tcatgcg (SEQ ID NO:76)

MRSAII DNA sequence:

tttagttgcagaaagaatttt**ctcaaagctagaactttgcttcactataagtattc**agtata aagaatatttcgctattatttacttgaaatgaaagactgcggaggctaactatgtcaaaaat catgaacctcattacttatgataagcttcttaaaacataacagcaattcacataaacctca tatgttctgatacattcaaaatccctttatgaagcggctgaaaaaaccgcatcatttatgat atgctt***ctccacgcataatcttaaatgctctgtacacttgttcaattaaca***caacccgcatc atttgatgtgggaatgtcattttgctgaatgatagtgcgtagttactgcgttgtaagacgtc cttgtgcaggc**cgtttgatccgccaatgacgaatacaaagtcgctttgcccttggg**tcatgc g (SEQ ID NO:77)

MRSAIII DNA sequence:

aaggtataat**ccaatatttcatatatgtaattcctccacatctca**ttaaattttaaattat
acacaacctaattttagttttatttatgatacgctt***ctccacgcataatcttaaatgctct***
gtacacttgttcaattaacacaacccgcatcatttgatgtgggaatgtcattttgctgaatg
atagtgcgtagttactgcgttgtaagacgtccttgtgcaggc**cgtttgatccgccaatgacg**
aatacaaagtcgctttgcccttgggtcatgcg (SEQ ID NO:78)

Example 2 Kinetics of an RPA reaction

A schematic of the RPA process is shown in Figure 12A. Recombinase/primer filaments scan template DNA for homologous sequences (red/blue). Following strand exchange the displaced strand is bound by gp32 (green), primers are extended by Bsu polymerase (blue). Repeated binding/extension events of opposing primers result in exponential DNA amplification.

The kinetics of recombinase/primer filament formation is shown in Figure 12B. In the presence of ATP uvsX (grey) binds cooperatively to oligonucleotides (red, top). Upon ATP hydrolysis the nucleoprotein complex disassembles (left) and uvsX can be replaced by gp32 (green, right). The presence of uvsY and Carbowax20M shifts the equilibrium in favor of recombinase loading.

The result of a typical RPA reaction is shown in Figure 12C which is a PAGE of RPA reactions using primers for STR markers. Genomic DNA from two individuals (1/2, father/son) served as template. Occasionally (D7S820, D16S539), low-level amounts of dimeric forms of full-length product can be observed (asterisks).

The ability to monitor RPA reaction in real time is shown in Figure 12D. In Figure 12D, a real-time RPA using primers for the *B. subtilis* SpoB locus was monitored by monitoring the fluorescence of a reaction. Fluorescence upon intercalation of SybrGreenI into nascent product is detected. *B. subtilis* DNA at $5x10^5$ (black), $5x10^4$ (red), $5x10^3$ (yellow), 500 (green) or 50 copies (purple) or water (blue) served as template. The onset of amplification depends linearly on the logarithm of the starting template copy number (see inset; time (midpoint of growth curve) versus log [template concentration]).

Example 3 Detection of RPA Amplicons Using Lateral Flow Strips

We devised a method of using lateral-flow-strip technology for the detection of RPA amplicon. This method uses specific antibodies to immobilize and detect complexes containing two antigenic labels (Figure 13A). Briefly, a target nucleic acid is amplified using two different oligonucleotide primers, wherein each primer comprises a different label or antigen. Thus, all generated amplicons would be linked to two labels or antigens (i.e., a double labeled amplicon).

To detect the presences of the double labeled amplicons, samples suspected of containing the amplicons a pad soaked in visible (gold) particles coupled to an antibody recognizing one of the two labels (in this case, the label is an antigen) (Figure 13C). The complexes then travel in a buffer stream through the membrane and an additional, immobilized antibody captures the second antigen (Id.). If the antigens are conjoined in a DNA duplex, a colored line appears at a defined location on the strip. In a variation of our probe detection system we produced such dual antigen complexes by coupling Biotin- and FAM-bearing oligonucleotides in RPA amplicons (Figure 3B). The 5'-biotinylated primer and its opposing counterpart ensure the efficient amplification of a target for probe binding. The probe, including a 5'-FAM label, an internal THF and a 3'-blocking group, is incised by Nfo upon binding, creating a 3'OH substrate for elongation by Bsu. The extension of the probe remnant stabilizes its interaction with the Biotin-labeled strand and produces an amplicon that contains both, Biotin and FAM. The THF/3'block prevents the production of Biotin/FAM containing primer artifacts, as processing of bona fide duplexes by Nfo adds a critical proofreading step. After application of the sample to the lateral-flow-strip Biotin/FAM-amplicons will create a visible signal on the FAM detection line, while RPA reactions that fail to generate a conjoined complex will not. We used a multiplex approach similar to the one employed in Figure 10E to detect 10 copies of each of the three MRSA alleles and distinguish them from MSSA (Figure 3C).

A number of research and clinical applications could benefit from employing RPA and the various detection methods disclosed herein. For example, RPA offers a significant breakthrough for the development of non-laboratory devices. When integrated with handheld instruments or entirely equipment free DNA detection systems, RPA will enable an easy-to-use testing system for a variety of pathogens as well as field kits for other applications.

Materials and methods

Proteins and DNA

Coding sequences for uvsx, uvsy, gp32, Bsu and Nfo were amplified from genomic DNA (DSMZ, Germany), fused to hexahistidine-tags (N-terminal for uvsY, Bsu and Nfo, C-terminal for uvsX and gp32) and cloned into suitable expression vectors. Overexpression and purification was done by standard protocols using Nickel-NTA resin (Qiagen).

Human DNA was purified from blood (Wizard-Genomic-purification-kit, Promega), *B. subtilis* DNA was from ATCC (USA), *S. aureus* DNAs were a gift from Jodi Lindsay. *S. aureus* alleles were EMRSA-3 (SCC*mec* type I; MRSAI), EMRSA-16 (MRSAII), EMRSA-1 (MRSAIII) and wild-type MSSA (*12*). See supplementary information for sequences.

RPA conditions

Reactions were performed at 37°C for 60min or as indicated. Standard conditions were 50mM Tris (pH 8.4), 80mM Potassium-acetate, 10mM Magnesium-acetate, 2mM DTT, 5% Carbowax20M, 200 µM dNTPs, 3mM ATP, 20mM Phosphocreatine, 100ng/µl Creatine-kinase, 20ng/µl Bsu. In contrast, MRSA amplifications were done at 50mM Tris (pH 7.9), 100mM Potassium-acetate, 14mM Magnesium-acetate; in the multiplex experiment Carbowax20M was at 5.5%. Concentrations of gp32/uxsX/uvsY (in ng/ul) were 600/200/60 (STR experiment), 600/120/30 (*B. subtilis* experiment) or 900/120/30 (MRSA experiments). Primers were employed at 300nM each, except in MRSA amplification, where 500nM sccIII, 100nM orfX (MRSAIII experiment) or 265nM sccI/II, 265nM sccIII, 70nM orfX (multiplex experiment) or 240nM BiosccI/ II, 240nM Bio-sccIII, 120nM orfX (lateral-flow-strip experiment) have been used. Reaction volumes were 20 µl, except for the STR experiment (40µl) and the *B. subtilis* experiment (50µl).

Real-time monitoring

Real-time RPA was performed in a plate-reader (BioTek Flx-800) in the presence of SybrGreenI (1:50000, Molecular Probes) or fluorophore/quencher probes (Eurogentec). Three probes were employed:

SATamra1 5'-tgttaattgaacaagtgtacagagcatt(T)a(H)ga(q1)tatgcgtggag-Biotin-3'

SATamra2 5'-tgttaattgagcaagtgtatagagcatt(T)a(H)ga(q2)tatgcgtggag-Biotin-3'

BSFlc 5'-catgattggatgaataagctgcagc(F)g(H)t(q3)aaaggaaactta-Biotin-3'

Here (T) is dT-TAMRA, (F) is dT-Fluorescein, (H) is THF, (q1) is dT-BHQ1, (q2) is dT-BHQ2, (q3) is dT-DDQ1. Probes were employed at 60nM SATamra1 (MRSAIII experiment) or at 45nM SATamra1, 45nM SATamra2, 60nM BSFlc (multiplex experiment). Nfo was used at 200ng/ul. Excitation/detection was at 485/525nm (SybrGreenI, BSFlc) or

530/575nm (SATamra1/2). Measurements were taken every 30sec or 45sec (multiplex experiment). Fluorescence probe data were normalised against water control and pre-amplification baseline adjusted. The logarithm of the read-out was plotted against reaction time.

Lateral-flow-strip detection

For lateral-flow-strip experiments two probes were used at 75nM each:

Lfs1 5'FAM-ccacatcaaatgatgcgggttgtgttaat(H)gaacaagtgtacagag-ddC-3'

Lfs2 5'FAM-ccacatcaaatgatgcgggttgtgttaat(H)gagcaagtgtatagag-ddC-3'

5'-biotinylated forms of sccI/II and sccIII were utilised as primers. For each reaction (20ul) 1ul was diluted with 5ul running buffer (PBS/3%Tween) and applied directly to HybriDetect-strips (Milenia) according to manufacturer instructions.

The result of the lateral flow strip detection is shown in Figure 13C. Reactions contained (left to right) 10 copies MRSAIII, 10 copies MRSAII, 10 copies MRSAI or 10000 copies MSSA (negative control) as template. Positive signals are generated in the first 3 reactions (arrowhead).

Example 4 Analysis of Optimal Conditions for RPA

RPA conditions

RPA relies on the establishment of a reaction environment that support the formation of recombinase-oligonucleotide complexes. Since the process is also ATPdependent (Formosa et al., 1986), it requires an energy regeneration system for sustained activity. In this experiment, we titrated key components of the RPA reaction mixture in order to determine their influence on amplification performance. The results are shown in Figure 14. Figure 14 shows polyacrylamide gel electrophoresis of RPA reactions using primers for the human Sry locus. Reactions were performed at 37°C for 120min and contained the primers sry3 and sry4 at 300 nM, 50mM Tris (pH 8.4), 80mM Potassium-acetate, 10mM Magnesium-acetate, 2mM DTT, 3mM ATP, 200μM dNTPs, 20mM Phosphocreatine, 100ng/μl Creatine-kinase, 5% Carbowax20M, 600ng/μl gp32, 200ng/μl uvsX, 60ng/μl uvsY and 20ng/μl Bsu, except when a given component was that under investigation. Optimal quantities of (Figure 14 **A**) gp32, (Figure 14 **B**) uvsY, (Figure 14 **C**) uvsX, (Figure 14 **D**) Carbowax20M, (Figure 14 **E**) ATP and (Figure 14 **F**) Bsu for effective amplification of this particular target were determined. **(G)** ADP-®-S and **(H)** ATP-©-S inhibit the reactions. 1500 copies/μl of the

human Y-chromosomal DNA served as template in 30ul reactions (per sample the equivalent of 10ul reaction volume was loaded on the gel).

RPA proved to work robustly over a relatively wide range of reagent concentrations. We found, however, that optimal reaction conditions varied between different primer pairs and therefore had to be defined individually.

Primer requirements

We used RPA to amplify of a wide range of targets. While the design of primers revealed no limitations on sequence composition itself, certain parameters have to be met for an oligonucleotide to be suitable for RPA. We investigated these parameters in the experiments shown in Figure 15. Figure 15 shows agarose gel electrophoresis of RPA reactions using primers for the human Apolipoprotein B locus. Primer ApoB4 was combined with opposing primers capable of generating products of the indicated sizes. Reactions were performed at 37°C for 120min and conditions used were 50mM Tris (pH 8.4), 80mM Potassiumacetate, 10mM Magnesium-acetate, 2mM DTT, 3mM ATP, 200µM dNTPs, 20mM Phosphocreatine, 100ng/µl Creatine-kinase, 5% Carbowax20M, 600ng/µl gp32, 125ng/µl uvsX, 25ng/µl uvsY, and 20ng/µl Bsu. 450 copies of human DNA were used as template in 30µl reactions (per sample the equivalent of 10ul reaction volume was loaded on the gel). Note that some hairpin-mediated product duplication occurred, converting some of the 300bp amplicon to 2x and 3x unit length (*). RPA failed to produce amplicons of 1500bp or more. This experiment shows that amplicon size under the conditions employed is limited to approximately 1kb.

Shown is polyacrylamide gel electrophoresis of RPA reactions using primers for the three independent loci in human genomic DNA (Apolipoprotein B, STR D18S51, Sry). Primers were 25, 28, or >31 bases, as indicated. Reactions were performed at 37°C for 120min. Conditions used were 50mM Tris/Cl pH 8.4, 80mM Potassium acetate, 10mM Magnesium-acetate, 2mM DTT, 3mM ATP, 200µM dNTPs, 20mM Phosphocreatine, 100ng/µl Creatine kinase, 5% Carbowax20M, 600ng/µl gp32, 200ng/µl uvsX and 60ng/µl uvsY, and 20ng/µl Bsu polymerase. 3000 copies of target served as template in 30ul reactions (per sample the equivalent of 10ul reaction volume was loaded on the gel). The finding that a primer length of >28 bases is required to support RPA is in good agreement with reports that investigated the ATP hydrolysis activity of uvsX-oligonucleotide filaments at different oligonucleotide sizes (See, Huletsky et al., 2004).

"The minimum length of a primer proved to be about 30 nucleotides (Figure 16). We observed variability in the performance of oligonucleotides that differ in sequences but are similar in length and position relative to their counterpart. The rules governing the influence of nucleotide sequence on the quality of a particular RPA primer are currently under investigation.

Control DNA

The wild-type *S. aureus* DNA (MSSA) (See, Enright et al., 2002; Huletsky et al., 2004) serving as a negative control in the experiment shown in 2C does act as a template for RPA when combined with the primer pair orfX/mssa (Figure 16).

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Claims

1. A RPA process of DNA amplification of a target nucleic acid molecule comprising a first and a second strand of DNA, comprising the steps of:

   (a) contacting a recombinase agent with a first and a second nucleic acid primer and a third extension blocked primer which comprises one or more noncomplementary or modified internal residue to form a first, second and third nucleoprotein primer;

   (b) contacting the first and second nucleoprotein primers to said double stranded target nucleic acid thereby forming a first double stranded structure between said first nucleoprotein primer and said first strand of DNA at a first portion of said first strand and a second double stranded structure between said second nucleoprotein primer and said second strand of DNA at a second portion of said second strand such that the 3' ends of said first nucleoprotein primer and said first nucleoprotein primer are oriented toward each other on the same target nucleic acid molecule with a third portion of target nucleic acid between said 3' ends;

   (c) extending the 3' end of said first nucleoprotein primer and second nucleoprotein primer with one or more polymerases and dNTPs to generate a first amplified target nucleic acid with an internal region comprising the third portion of nucleic acid;

   (d) contacting said amplified target nucleic acid to said third nucleoprotein primer to form a third double stranded structure at the third portion of said amplified target nucleic acid in the presences of a nuclease; wherein said nuclease specifically cleaves said noncomplementary internal residue only after the formation of said third double stranded structure to form a third 5' primer and a third 3' extension blocked primer;

   (e) extending the 3' end of said third 5' primer with one or more polymerase and dNTP to generate a second double stranded amplified nucleic acid which comprises said first nucleic acid primer and said third 5' primer;

   (f) continuing the reaction through repetition of (b) and (e) until a desired degree of the second double stranded amplified nucleic acid is reached.

2. The process of claim 1 wherein the first double stranded structure is part of a first D-loop and wherein said second double stranded structure is part of a second D-loop.

3. The process of claim 1 wherein said nuclease is a DNA glycosylase or AP endonuclease.

4. The process of claim 1 wherein said modified internal residue is a uracil or inosine residue.
5. The process of claim 4 wherein the nuclease recognizes the uracil or inosine residue cleaves said third extension blocked primer at the uracil or inosine residue.
6. The process of claim 1 wherein the nuclease recognizes a base mismatch between the noncomplementary base of said third extension blocked primer and said target nucleic acid and cleaves said third extension blocked primer at said noncomplementary base.
7. The process of claim 1 wherein said nuclease is selected from the group consisting of fpg, Nth, MutY, MutS, MutM, E. coli MUG, human MUG, human Oggl, vertebrate Nei-like (Neil) glycosylases, uracil glycosylase, hypoxanthine-DNA glycosylase, and functional analogs thereof.
8. The process of claim 1 wherein said nuclease is E. coli Nfo or E. coli exonuclease III and wherein the modified residue is a tetrahydrofuran residue or carbon linker.
9. The process of claim 1 wherein the modified internal base is selected from the group consisting of 8-oxoguanine, thymine glycol, an abasic site mimic.
10. The process of claim 9 wherein the abasic site mimic is a tetrahydrofuran residue or D-spacer.
11. The process of claim 1 wherein said third extension blocked primer comprises a blocked 3' residue which is resistant to extension by DNA polymerase.
12. The process of claim 11 wherein the blocked 3' residue comprises a blocking moiety which prevents the extension of the primer by polymerase.
13. The process of claim 12 wherein the blocking moiety is attached to the 3' or 2' site of the 3' residue sugar.
14. The process of claim 12 wherein the blocking moiety is a detectable label.
15. The process of claim 14 wherein said detectable label is selected from the group consisting of a fluorophore, an enzyme, a quencher, an enzyme inhibitor, a radioactive label, a member of a binding pair, and a combination thereof.
16. The process of claim 11 wherein the blocked 3' residue is a dideoxy nucleotide.
17. The process of claim 1 wherein said first nucleic acid primer comprises a first detectable lable and said third extension blocked primer comprises a second detectable label.
18. The process of claim 17 wherein said first and second detectable label are different and the production of said second double stranded amplified nucleic acid is monitored

by detecting the presence of the first and second detectable label on a single double stranded DNA molecule.

19. The process of claim 18 wherein the production of said second double stranded amplified nucleic acid is detected by a sandwich assay wherein a first antibody binds said first detectable label and a second antibody binds said second detectable label.
20. The process of claim 1 wherein the third extension blocked primer further comprises one or more detectable labels.
21. The process of claim 20 wherein said process further comprises the step of monitoring the progress of the RPA reaction by detecting said detectable label on the third extension blocked primer.
22. The process of claim 20 wherein said detectable label is selected from the group consisting of a fluorophore, an enzyme, a quencher, an enzyme inhibitor, a radioactive label, one member of a binding pair and a combination thereof.
23. The process of claim 22 in which the fluorophore is attached to the third extension blocked primer by a fluorophore-dT amidite residue.
24. The process of claim 22 in which the quencher is attached to the third extension blocked primer by a quencher-dT amidite residue.
25. The process of claim 22 wherein said third extension blocked primer comprises a fluorophore and a quencher.
26. The process of claim 25 wherein said fluorophore and quencher are separated by between 0 to 2 bases.
27. The process of claim 25 wherein said fluorophore and quencher are separated by between 0 to 5 bases.
28. The process of claim 25 wherein said fluorophore and quencher are separated by between 0 to 8 bases.
29. The process of claim 25 wherein said fluorophore and quencher are separated by between 0 to 10 bases.
30. The process of claim 25 wherein said fluorophore and quencher are separated by a greater distance when the extension blocked primer is unhybridized than when the extension blocked primer is hybridized to the target nucleic acid.
31. The process of claim 25 wherein the fluorophore or the quencher is attached to the noncomplementary or modified internal residue and wherein the fluorophore and quencher are separated following cleavage of the modified internal base by the nuclease.

32. The process of claim 25 in which the fluorophore is selected from the group of fluorescein, FAM, TAMRA.
33. The process of claim 25 in which the quencher is a dark quencher.
34. The process of claim 33 wherein said dark quencher is selected from the group consisting of Dark Quencher 1, Dark Quencher 2, Black Hole Quencher 1 and Black Hole Quencher 2.
35. The process of claim 1 wherein the first primer, second primer or third extention blocked primer is 12 to 30 residues in length.
36. The process of claim 1 wherein the first primer, second primer or third extention blocked primer 12 to 40 residues in length.
37. The process of claim 1 wherein the first primer, second primer or third extention blocked primer 12 to 60 residues in length.
38. The process of claim 1 wherein said process is performed at a temperature of between 14°C and 21°C.
39. The process of claim 1 wherein said process is performed at a temperature of between 21°C and 25°C.
40. The process of claim 1 wherein said process is performed at a temperature of between 25°C and 30°C.
41. The process of claim 1 wherein said process is performed at a temperature of between 30°C and 37°C.
42. The process of claim 1 wherein said process is performed at a temperature of between40°C and 43°C.
43. The process of claim 1 wherein said process amplifies at least the third portion of said target nucleic acid at least 10^7 folds.
44. The process of claim 1 wherein said process is performed in the presence of 1% to 12% PEG.
45. The process of claim 1 wherein said process is performed in the presence of 6% to 8% PEG.
46. The process of claim 1, wherein said dNTP comprises dUTP and wherein said RPA process is performed in the presence of uracil glycosylase for a first period of less than 20 minutes and wherein said process is performed in the presence of uracil glycosylase inhibitor after said first period.
47. The process of claim 46 wherein said process is performed without temperature based inactivation of said uracil glycosylase.

48. The process of claim 46 wherein said uracil glycosylase inhibitor is *Bacillus subtilis* phages PBS1 uracil glycosylase inhibitor or *Bacillus subtilis* phages PBS2 uracil glycosylase inhibitor.

49. The process of claim 46 wherein said dNTP consists of dATP, dUTP, dCTP and dGTP.

50. The process of claim 46 wherein said dNTP does not contain dTTP.

51. A multiplex process of RPA comprising the steps of performing more than one RPA process on one or more double stranded target nucleic acid in one reaction wherein each process comprise the following steps:

(a) contacting a recombinase agent with a first and a second nucleic acid primer and a third extension blocked primer which comprises one or more noncomplementary or modified internal residue to form a first, second and third nucleoprotein primer;

(b) contacting the first and second nucleoprotein primers to said double stranded target nucleic acid thereby forming a first double stranded structure between said first nucleoprotein primer and said first strand of DNA at a first portion of said first strand and a second double stranded structure between said second nucleoprotein primer and said second strand of DNA at a second portion of said second strand such that the 3' ends of said first nucleoprotein primer and said first nucleoprotein primer are oriented toward each other on the same target nucleic acid molecule with a third portion of target nucleic acid between said 3' ends;

(c) extending the 3' end of said first nucleoprotein primer and second nucleoprotein primer with one or more polymerases and dNTPs to generate a first amplified target nucleic acid with an internal region comprising the third portion of nucleic acid;

(d) contacting said amplified target nucleic acid to said third nucleoprotein primer to form a third double stranded structure at the third portion of said amplified target nucleic acid in the presences of a nuclease; wherein said nuclease specifically cleaves said noncomplementary internal residue only after the formation of said third double stranded structure to form a third 5' primer and a third 3' extension blocked primer;

(e) extending the 3' end of said third 5' primer with one or more polymerase and dNTP to generate a second double stranded amplified nucleic acid which comprises said first nucleic acid primer and said third 5' primer;

(f) continuing the reaction through repetition of (b) and (e) until a desired degree of the second double stranded amplified nucleic acid is reached;

wherein each RPA process is performed with a different combination of said first and second nucleic acid primer and wherein each process is performed with the same third extension blocked primer.

52. The method of claim 51 wherein said more than one RPA processes comprises at least 2 separate RPA processes.

53. The method of claim 51 wherein said more than one RPA processes comprises at least 4 separate RPA processes.

54. The method of claim 51 wherein said more than one RPA processes comprises at least 5 separate RPA processes.

55. The method of claim 51 wherein said more than one RPA processes comprises at least 7 separate RPA processes.

56. The method of claim 51 wherein said more than one RPA processes comprises at least 10 separate RPA processes.

57. The process of claim 51 wherein said modified internal residue is a uracil or inosine residue.

58. The process of claim 51 further comprising the step of detecting the formation of said second double stranded amplified nucleic acid to determine the cumulative amplification of any of said more than one RPA process.

59. The process of claim 51 wherein the first nucleic acid primer of each RPA process is labeled with the same first detectable label, wherein said third extension blocked primer is labeled with a second detectable label, and wherein said detecting step comprises detecting a double stranded nucleic acid comprising both said first detectable label and said second detectable label.

60. The process of claim 59 wherein the production of said second double stranded amplified nucleic acid is detected by a sandwich assay wherein a first antibody binds said first detectable label and a second antibody binds said second detectable label.

61. The process of claim 51 wherein said nuclease is a DNA glycosylase or AP endonuclease.

62. The process of claim 51 wherein the nuclease recognizes a base mismatch between the noncomplementary base of said third extension blocked primer and said target nucleic acid and cleaves said third extension blocked primer at said noncomplementary base.

63. The process of claim 51 wherein said nuclease is selected from the group consisting of fpg, Nth, MutY, MutS, MutM, E. coli MUG, human MUG, human Oggl, vertebrate Nei-like (Neil) glycosylases, uracil glycosylase, hypoxanthine-DNA glycosylase, and functional analogs thereof.

64. The process of claim 51 wherein said nuclease is E. coli Nfo or E. coli exonuclease III and wherein the modified residue is a tetrahydrofuran residue or carbon linker.

65. The process of claim 51 wherein the modified internal base is selected from the group consisting of 8-oxoguanine, thymine glycol, or an abasic site mimic.

66. or The process of claim 65 wherein the abasic site mimic is a tetrahydrofuran residue or D-spacer.

67. The process of claim 66 wherein the abasic site mimic is a tetrahydrofuran residue or D-spacer.

68. The process of claim 51 wherein said third extension blocked primer comprises a blocked 3' residue which is resistant to extension by DNA polymerase.

69. The process of claim 68 wherein the blocked 3' residue comprises a blocking moiety which prevents the extension of the primer by polymerase.

70. The process of claim 69 wherein the blocking moiety is attached to the 3' or 2' site of the 3' residue sugar.

71. The process of claim 70 wherein the blocked 3' residue is a dideoxy nucleotide.

72. A RPA process of DNA amplification of a double stranded target nucleic acid molecule comprising a first and a second strand of DNA, comprising the steps of:

(a) contacting a recombinase agent with a first and a second nucleic acid primer to form a first and a second nucleoprotein primer;

(b) contacting the first and second nucleoprotein primers to said double stranded target nucleic acid thereby forming a first double stranded structure between said first nucleoprotein primer and said first strand of DNA at a first portion of said first strand and a second double stranded structure between said second nucleoprotein primer and said second strand of DNA at a second portion of said second strand such that the 3' ends of said first nucleoprotein primer and said first nucleoprotein primer are oriented toward each other on the same double stranded target nucleic acid molecule;

(c) extending the 3' end of said first nucleoprotein primer and second nucleoprotein primer with one or more polymerases and dNTPs and dUTP to generate an amplified target nucleic acid molecule;

(d) continuing the reaction through repetition of (b) and (c) for a first period of no more than 20 minutes in the presence of uracil glycosylase;

(e) continuing the reaction through repetition of (b) and (c) in the presence of an uracil glycosylase inhibitor until a desired degree of amplification is reached.

73. The process of claim 72 wherein said first period is less than or equal to 10 minutes.

74. The process of claim 72 wherein said first period is less than or equal to 5 minutes.

75. The process of claim 72 wherein said first period is less than or equal to 2 minutes.

76. The process of claim 72 wherein said uracil glycosylase inhibitor is *Bacillus subtilis* phages PBS1 uracil glycosylase inhibitor or *Bacillus subtilis* phages PBS2 uracil glycosylase inhibitor.

77. The process of claim 72 wherein said dNTP consists of dATP, dUTP, dCTP and dGTP.

78. The process of claim 72 wherein said dNTP does not contain dTTP.

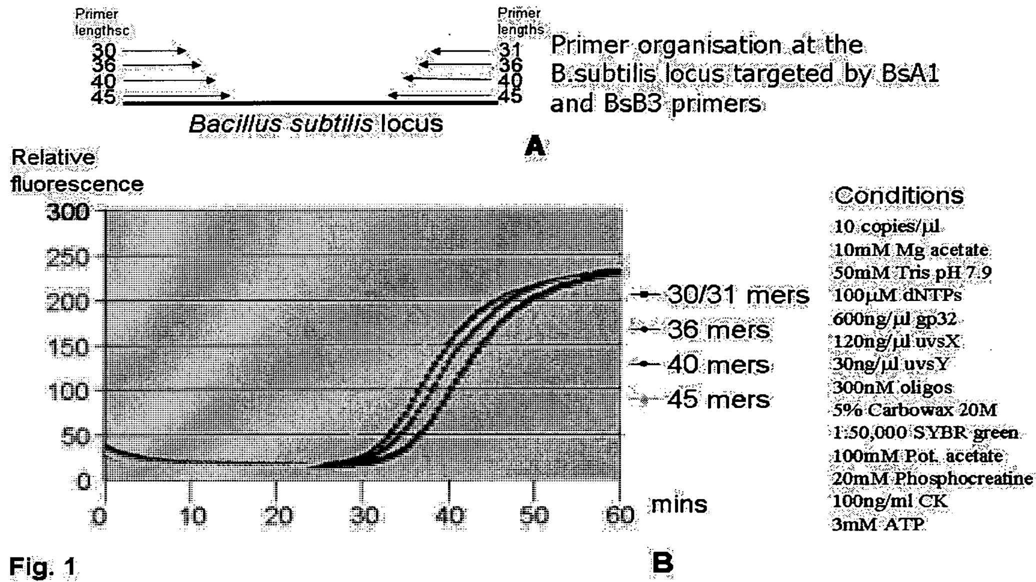


Fig. 1

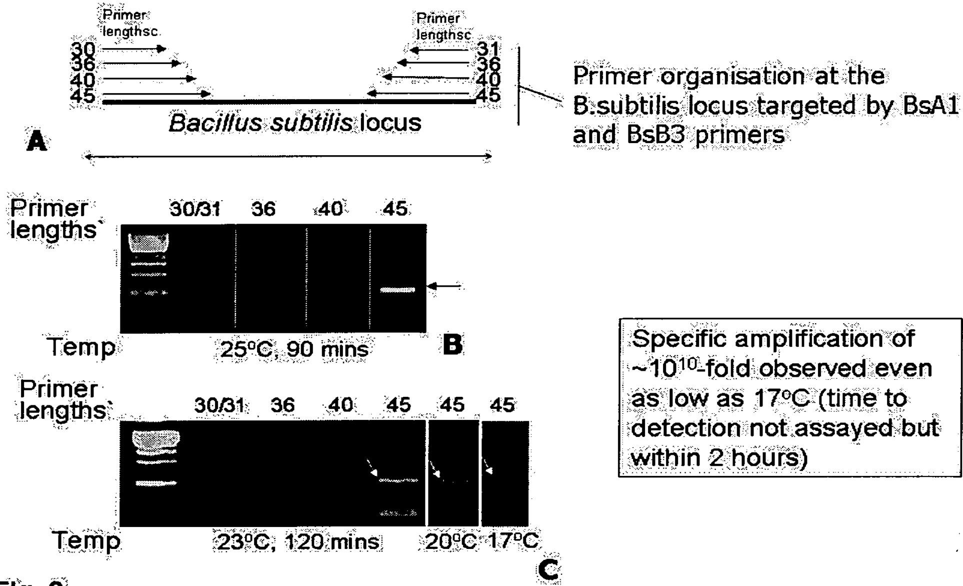


Fig. 2

Low temperature RPA demonstrates slower kinetics – PEG can be optimised

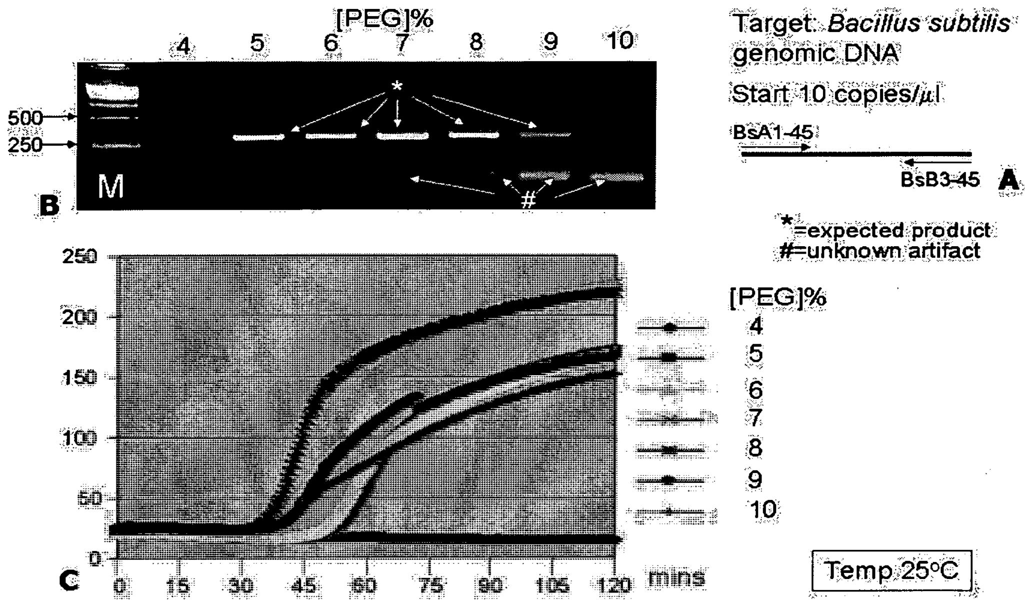


Fig. 3

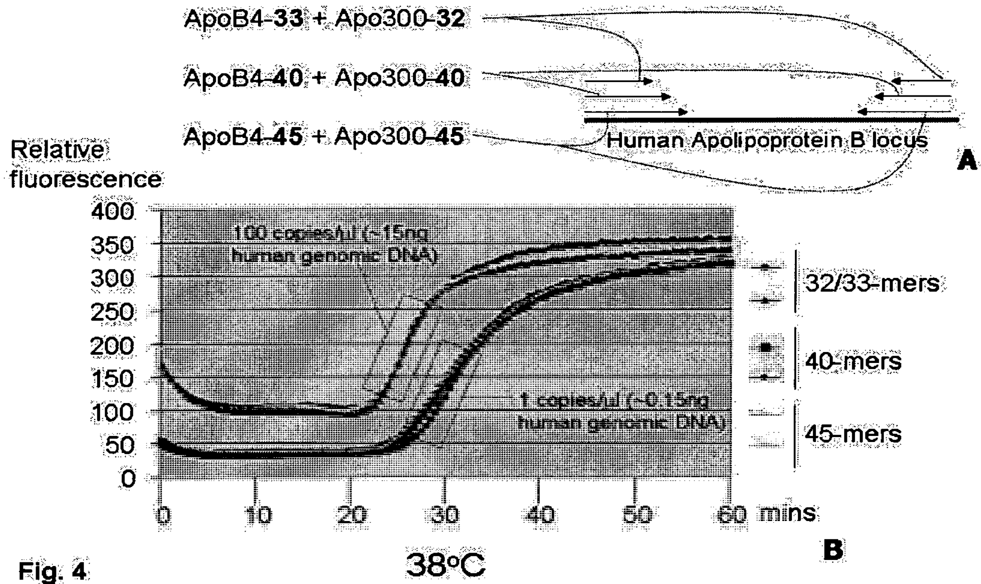


Fig. 4

Apolipoprotein B primers Apo300 and ApoB4 are 'fast' primers even when not 3' lengthened and operate at 'room temperature' (25°C)

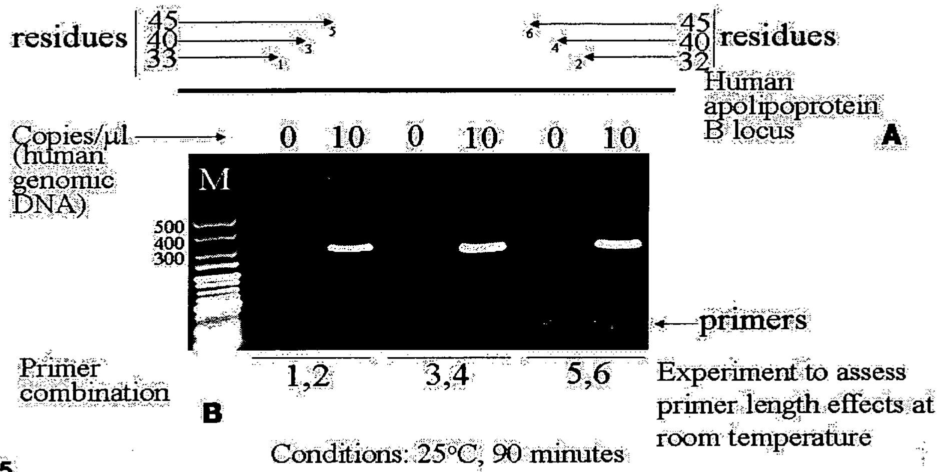
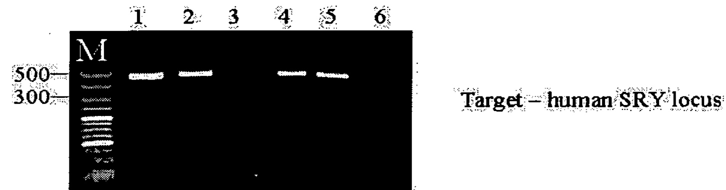


Fig. 5

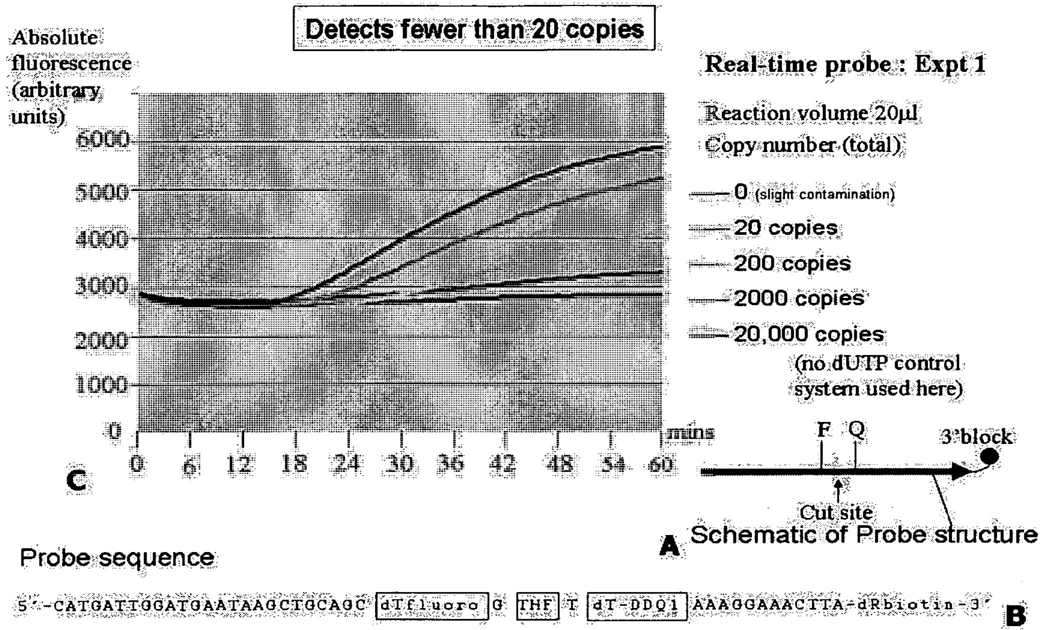
Carry-over contamination control using *E.coli* UNG & UNG inhibitor



1. Template
2. Template + UNG (5 minutes) + UNG Inhibitor (after 5 minutes)
3. Template + contaminating DNA (containing dUTP) + UNG
4. Template + contaminating DNA (containing dUTP) + UNG(5mins) + UNG Inhibitor
5. Contaminating DNA (containing dUTP)
6. Contaminating DNA (containing dUTP) + UNG(5mins) + UNG Inhibitor

Fig. 6

Fig. 7



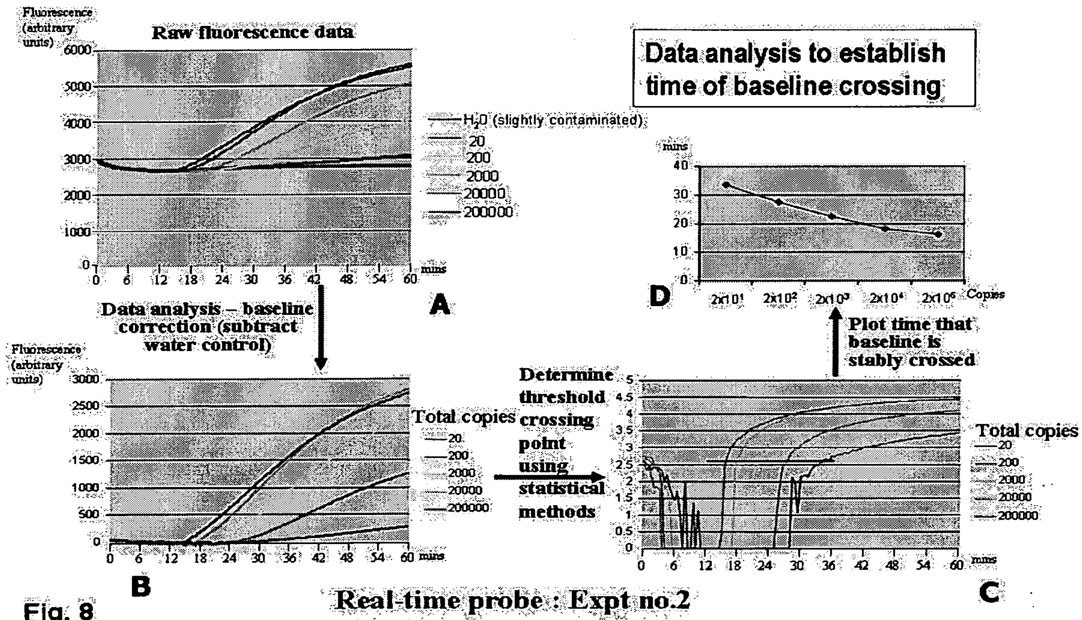


Fig. 8

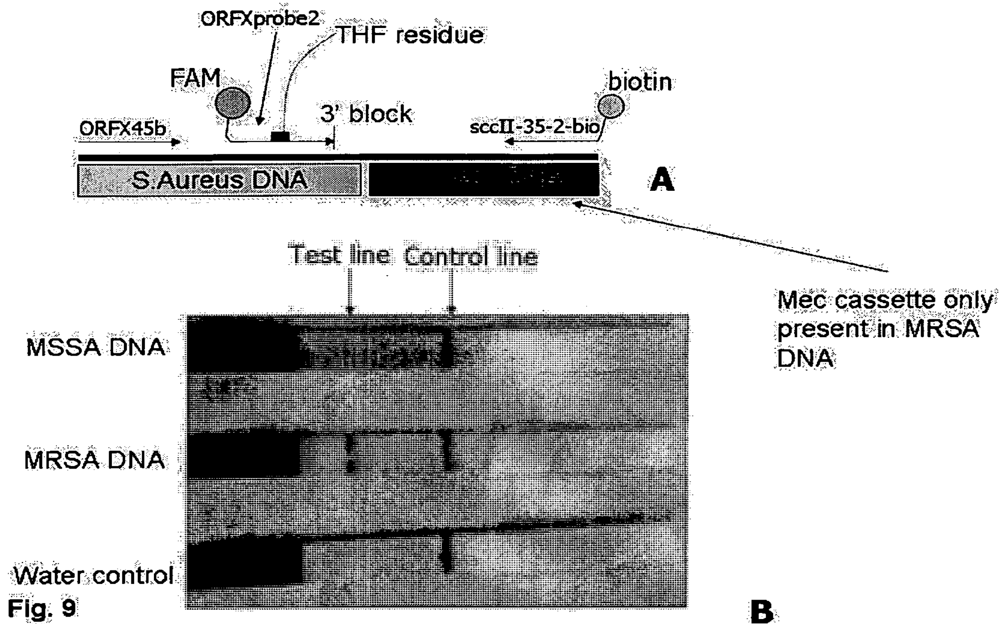


Fig. 9

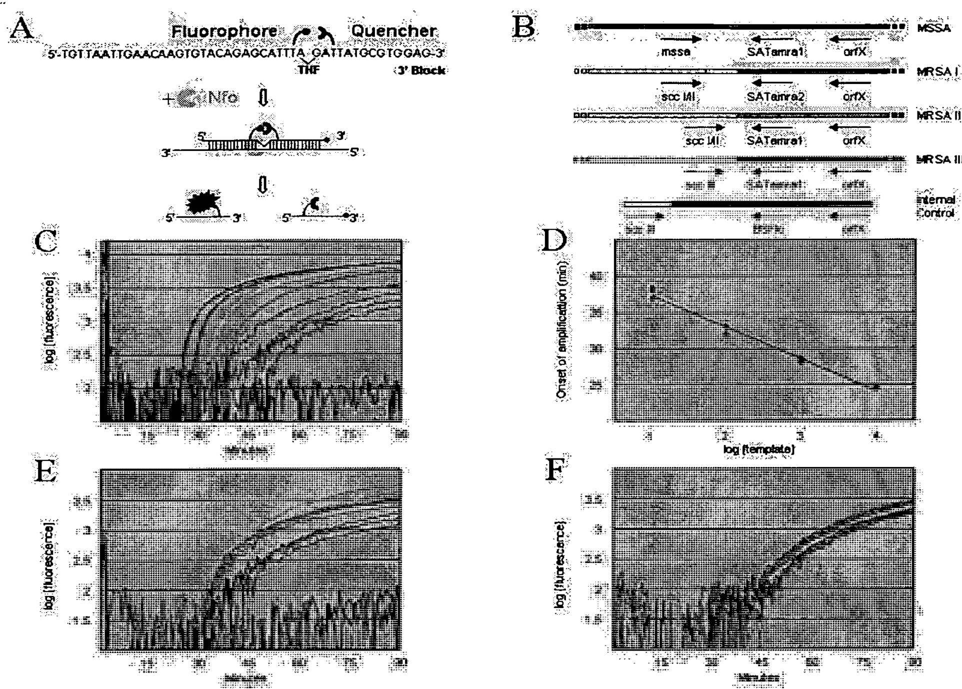


Figure 10

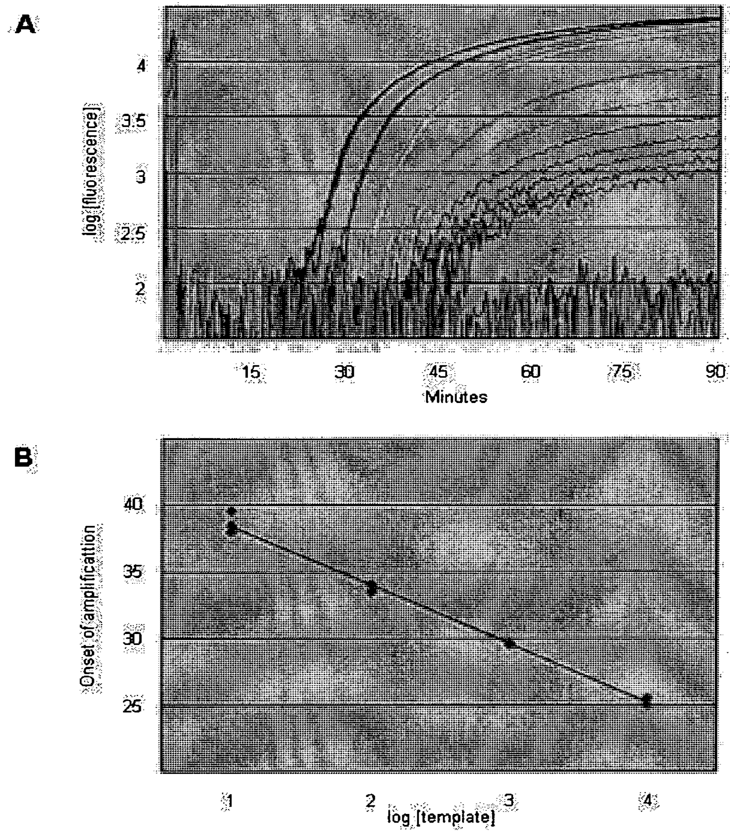


Figure 11

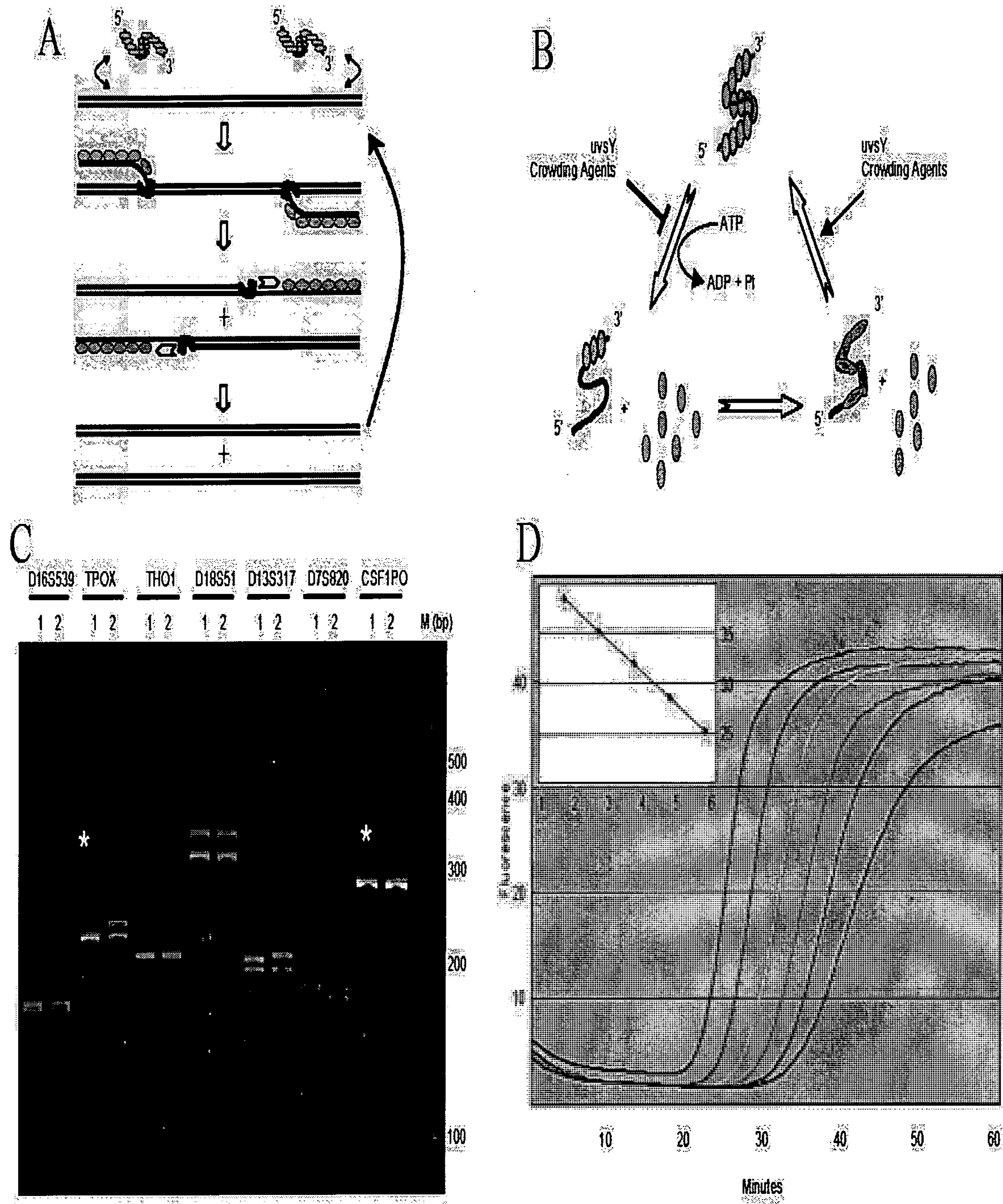


Figure 12

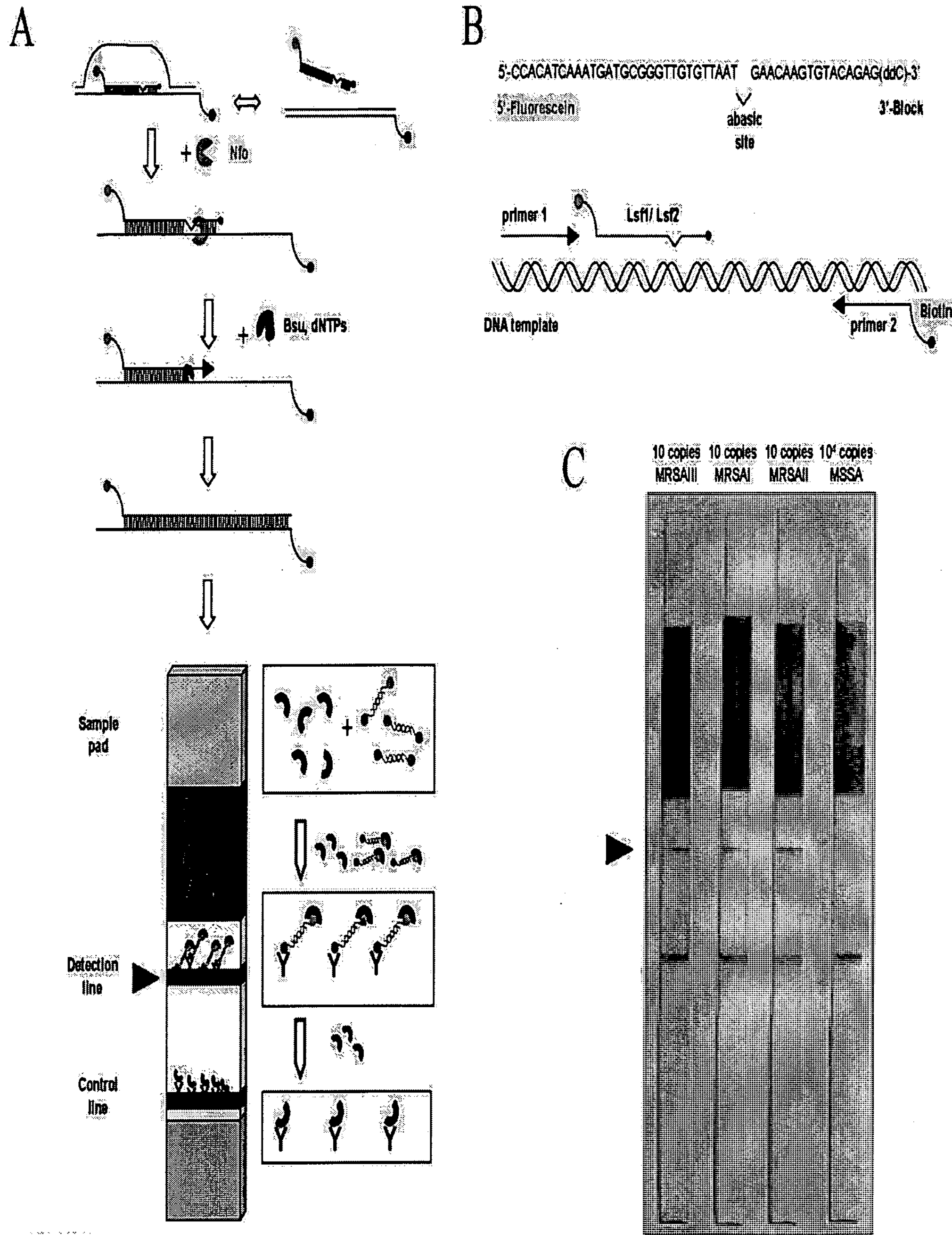


Figure 13

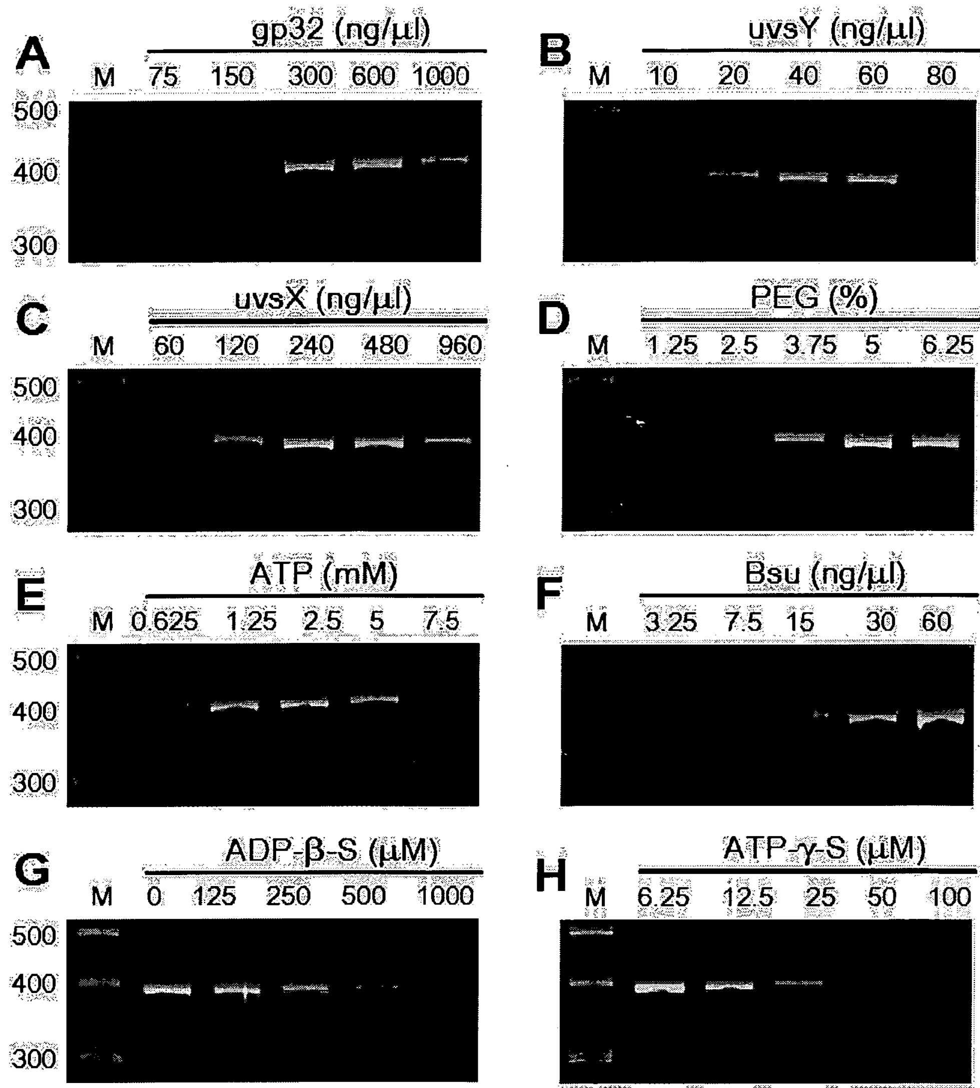
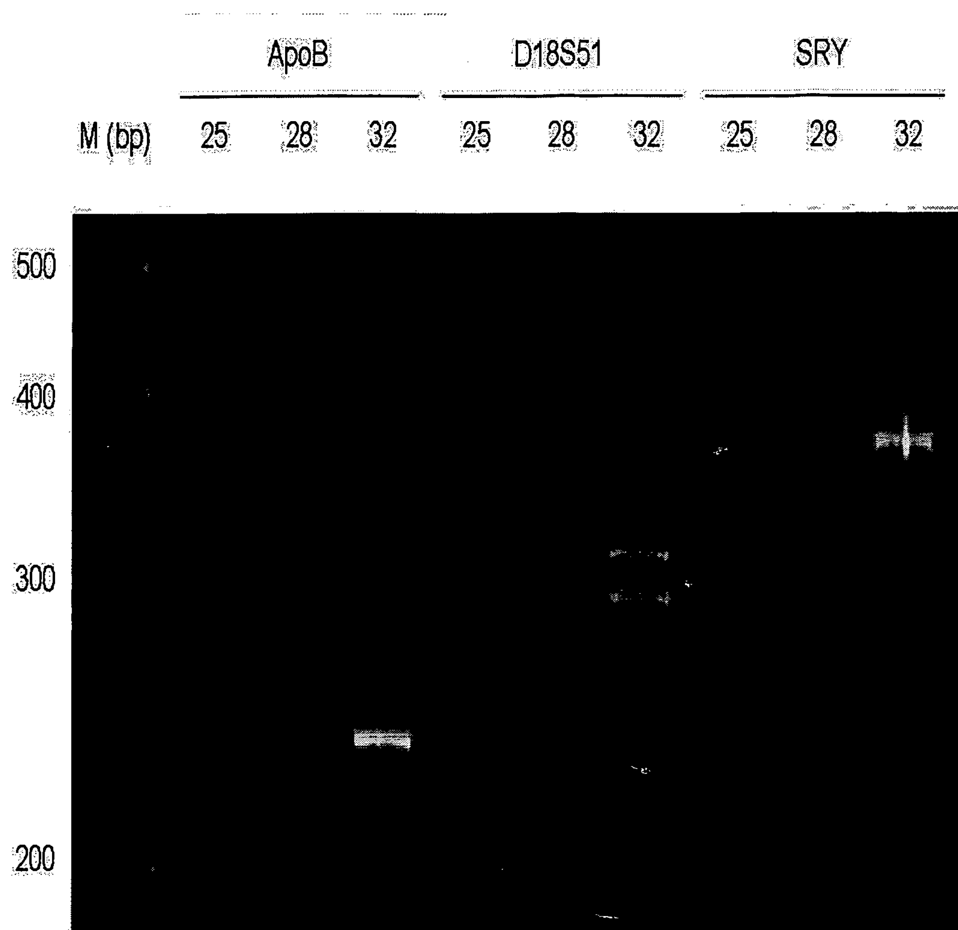


Figure 14



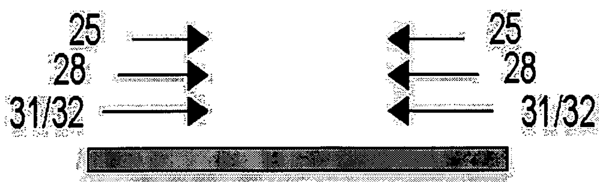


Figure 15

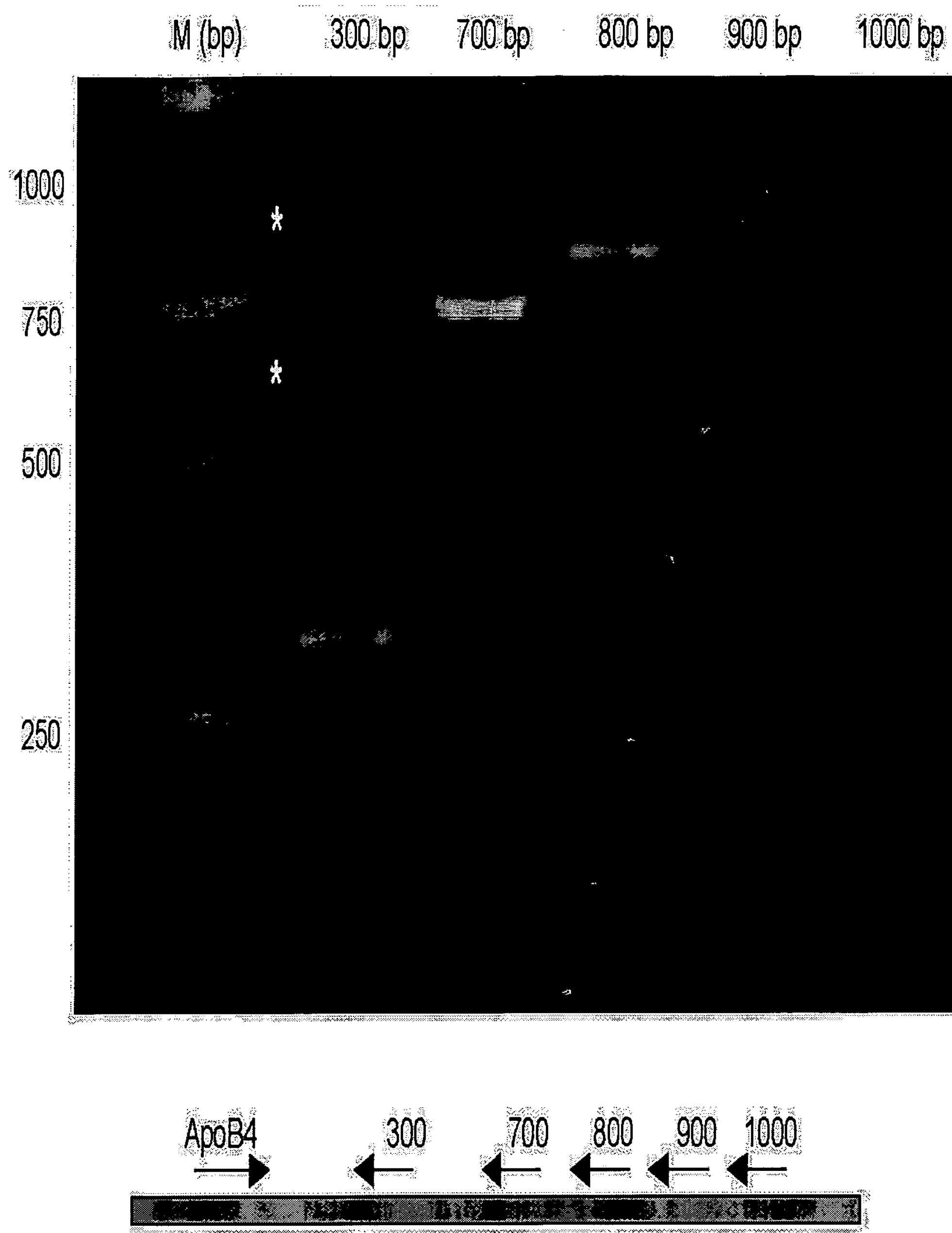


Figure 16

(12) INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(19) World Intellectual Property Organization
International Bureau

PCT

(43) International Publication Date
30 August 2007 (30.08.2007)

(10) International Publication Number
WO 2007/096702 A3

(51) International Patent Classification:
C12Q 1/68 (2006.01)

(21) International Application Number: PCT/IB2006/004113

(22) International Filing Date: 25 July 2006 (25.07.2006)

(25) Filing Language: English

(26) Publication Language: English

(30) Priority Data:
60/702,533 25 July 2005 (25.07.2005) US
60/728,424 18 October 2005 (18.10.2005) US

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(72) Inventors; and
(75) Inventors/Applicants (*for US only*): **PIEPENBURG, Olaf** [DE/GB]; c/o ASM Scientific Ltd., Meditrina, Babraham Research Campus, Babraham, Cambridge CB22 3AT (GB). **WILLIAMS, Colin, H.** [GB/GB]; c/o ASM Scientific Ltd., Meditrina, Babraham Research Campus, Babraham, Cambridge CB22 3AT (GB). **ARMES, Niall, A.** [GB/GB]; 140 Long Lane, London N3 2HX (GB).

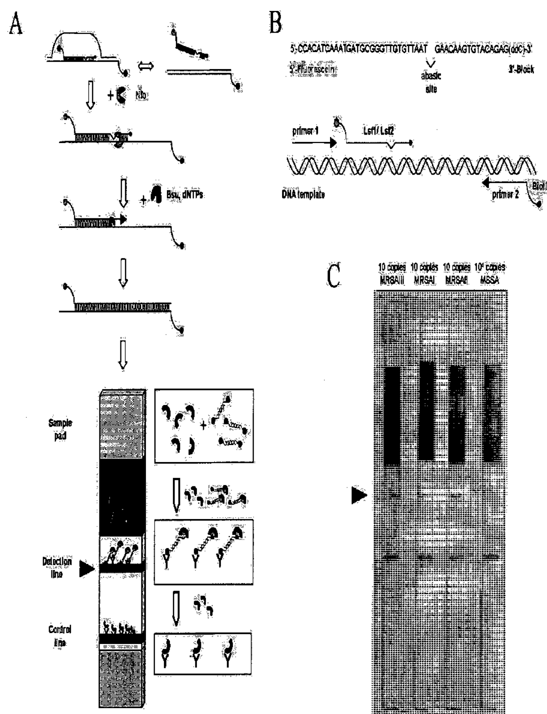
(74) Agent: **CRUMP, Julian, Richard, John**; Mintz Levin Cohn Ferris Glovsky & Popeo Intellectual Property LLP, The Rectory, 9 Ironmonger Lane, London EC2V 8EY (GB).

(81) Designated States (*unless otherwise indicated, for every kind of national protection available*): AE, AG, AL, AM, AT, AU, AZ, BA, BB, BG, BR, BW, BY, BZ, CA, CH, CN, CO, CR, CU, CZ, DE, DK, DM, DZ, EC, EE, EG, ES, FI, GB, GD, GE, GH, GM, HN, HR, HU, ID, IL, IN, IS, JP, KE, KG, KM, KN, KP, KR, KZ, LA, LC, LK, LR, LS, LT, LU, LV, LY, MA, MD, MG, MK, MN, MW, MX, MZ, NA, NG, NI, NO, NZ, OM, PG, PH, PL, PT, RO, RS, RU, SC, SD, SE, SG, SK, SL, SM, SY, TJ, TM, TN, TR, TT, TZ, UA, UG, US, UZ, VC, VN, ZA, ZM, ZW.

(84) Designated States (*unless otherwise indicated, for every kind of regional protection available*): ARIPO (BW, GH, GM, KE, LS, MW, MZ, NA, SD, SL, SZ, TZ, UG, ZM,

[Continued on next page]

(54) Title: METHODS FOR MULTIPLEXING RECOMBINASE POLYMERASE AMPLIFICATION



(57) Abstract: This disclosure provides for methods and reagents for rapid multiplex RPA reactions and improved methods for detection of multiplex RPA reaction products. In addition, the disclosure provides new methods for eliminating carryover contamination between RPA processes.

WO 2007/096702 A3

ZW), Eurasian (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European (AT, BE, BG, CH, CY, CZ, DE, DK, EE, ES, FI, FR, GB, GR, HU, IE, IS, IT, LT, LU, LV, MC, NL, PL, PT, RO, SE, SI, SK, TR), OAPI (BF, BJ, CF, CG, CI, CM, GA, GN, GQ, GW, ML, MR, NE, SN, TD, TG).

Published:
— *with international search report*
— *before the expiration of the time limit for amending the claims and to be republished in the event of receipt of amendments*

(88) Date of publication of the international search report:
24 July 2008

INTERNATIONAL SEARCH REPORT

International application No
PCT/IB2006/004113

A. CLASSIFICATION OF SUBJECT MATTER
INV. C12Q1/68

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
C12Q

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

EPO-Internal, WPI Data, BIOSIS

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	WO 03/072805 A (ASM SCIENT INC [US]; ARMES NIALL A [GB]; STEMPLE DEREK L [GB]) 4 September 2003 (2003-09-04) the whole document	
A	NADEAU J G ET AL: "REAL-TIME, SEQUENCE-SPECIFIC DETECTION OF NUCLEIC ACIDS DURING STRAND DISPLACEMENT AMPLIFICATION" ANALYTICAL BIOCHEMISTRY, ACADEMIC PRESS INC. NEW YORK, vol. 276, no. 2, 15 December 1999 (1999-12-15), pages 177-187, XP000906307 ISSN: 0003-2697 the whole document	

-/--

[X] Further documents are listed in the continuation of Box C.

[X] See patent family annex.

* Special categories of cited documents :

"A" document defining the general state of the art which is not considered to be of particular relevance
"E" earlier document but published on or after the international filing date
"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)
"O" document referring to an oral disclosure, use, exhibition or other means
"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.
"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
15 May 2008	30/05/2008

Name and mailing address of the ISA/	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL – 2280 HV Rijswijk Tel. (+31-70) 340-2040, Tx. 31 651 epo nl, Fax: (+31-70) 340-3016	Cornelis, Karen

Form PCT/ISA/210 (second sheet) (April 2005)

INTERNATIONAL SEARCH REPORT

International application No
PCT/IB2006/004113

C(Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	HARVEY J J ET AL: "Characterization and applications of CataCleave probe in real-time detection assays" ANALYTICAL BIOCHEMISTRY, ACADEMIC PRESS INC. NEW YORK, vol. 333, no. 2, 15 October 2004 (2004-10-15), pages 246-255, XP004573012 ISSN: 0003-2697 the whole document	
A	GINOCCHIO: "Life beyon PCR: Alternative target amplification technologies for the diagnosis of infectious diseases, part II" CLINICAL MICROBIOLOGY NEWSLETTER, vol. 26, September 2004 (2004-09), pages 129-136, XP002480278 the whole document	
P,X	WO 2005/118853 A (ASM SCIENT INC [GB]; PIEPENBURG OLAF [GB]; WILLIAMS COLIN H [GB]; ARME) 15 December 2005 (2005-12-15) claim 175	72-78

Form PCT/ISA/210 (continuation of second sheet) (April 2005)

INTERNATIONAL SEARCH REPORT

Information on patent family members

International application No
PCT/IB2006/004113

Patent document cited in search report		Publication date	Patent family member(s)			Publication date
WO 03072805	A	04-09-2003	AU	2003215391	A1	09-09-2003
			CA	2476481	A1	04-09-2003
			EP	1499738	A2	26-01-2005
			JP	2005518215	T	23-06-2005
WO 2005118853	A	15-12-2005	AU	2005250233	A1	15-12-2005
			CA	2569512	A1	15-12-2005
			EP	1759012	A2	07-03-2007
			JP	2008500831	T	17-01-2008

Form PCT/ISA/210 (patent family annex) (April 2005)

Electronic Acknowledgement Receipt	
EFS ID:	23650681
Application Number:	14067620
International Application Number:	
Confirmation Number:	4288
Title of Invention:	Nicking and Extension Amplification Reaction for the Exponential Amplification of Nucleic Acids
First Named Inventor/Applicant Name:	Brian K. Maples
Customer Number:	26161
Filer:	Ian J.S. Lodovice/Mary Florczak
Filer Authorized By:	Ian J.S. Lodovice
Attorney Docket Number:	30171-0025002 / ITI-001
Receipt Date:	30-SEP-2015
Filing Date:	30-OCT-2013
Time Stamp:	14:59:40
Application Type:	Utility under 35 USC 111(a)

Payment information:

Submitted with Payment	no

File Listing:

Document Number	Document Description	File Name	File Size(Bytes)/ Message Digest	Multi Part /.zip	Pages (if appl.)
1	Information Disclosure Statement (IDS) Form (SB08)	301710025002IDS.pdf	173376 f3dd49bd5a84e37125e39a870d45bdbafc8e9517	no	2

Warnings:

Information:

This is not an USPTO supplied IDS fillable form					
2	Foreign Reference	WO2007096702.pdf	5109308 8d8b2b5ddee374b70805f4d37b704baf520f7efe	no	83
Warnings:					
Information:					
		Total Files Size (in bytes):	5282684		

This Acknowledgement Receipt evidences receipt on the noted date by the USPTO of the indicated documents, characterized by the applicant, and including page counts, where applicable. It serves as evidence of receipt similar to a Post Card, as described in MPEP 503.

New Applications Under 35 U.S.C. 111
If a new application is being filed and the application includes the necessary components for a filing date (see 37 CFR 1.53(b)-(d) and MPEP 506), a Filing Receipt (37 CFR 1.54) will be issued in due course and the date shown on this Acknowledgement Receipt will establish the filing date of the application.

National Stage of an International Application under 35 U.S.C. 371
If a timely submission to enter the national stage of an international application is compliant with the conditions of 35 U.S.C. 371 and other applicable requirements a Form PCT/DO/EO/903 indicating acceptance of the application as a national stage submission under 35 U.S.C. 371 will be issued in addition to the Filing Receipt, in due course.

New International Application Filed with the USPTO as a Receiving Office
If a new international application is being filed and the international application includes the necessary components for an international filing date (see PCT Article 11 and MPEP 1810), a Notification of the International Application Number and of the International Filing Date (Form PCT/RO/105) will be issued in due course, subject to prescriptions concerning national security, and the date shown on this Acknowledgement Receipt will establish the international filing date of the application.

UNITED STATES PATENT AND TRADEMARK OFFICE

UNITED STATES DEPARTMENT OF COMMERCE
United States Patent and Trademark Office
Address: COMMISSIONER FOR PATENTS
P.O. Box 1450
Alexandria, Virginia 22313-1450
www.uspto.gov

APPLICATION NO.	FILING DATE	FIRST NAMED INVENTOR	ATTORNEY DOCKET NO.	CONFIRMATION NO.
14/067,620	10/30/2013	Brian K. Maples	30171-0025002 / ITI-001	4288

26161 7590 09/21/2015
FISH & RICHARDSON P.C. (BO)
P.O. BOX 1022
MINNEAPOLIS, MN 55440-1022

EXAMINER	
BERTAGNA, ANGELA MARIE	

ART UNIT	PAPER NUMBER
1637	

NOTIFICATION DATE	DELIVERY MODE
09/21/2015	ELECTRONIC

Please find below and/or attached an Office communication concerning this application or proceeding.

The time period for reply, if any, is set in the attached communication.

Notice of the Office communication was sent electronically on above-indicated "Notification Date" to the following e-mail address(es):

PATDOCTC@fr.com

PTOL-90A (Rev. 04/07)

Applicant-Initiated Interview Summary	**Application No.** 14/067,620	**Applicant(s)** MAPLES ET AL.	
	Examiner Angela M. Bertagna	**Art Unit** 1637	

All participants (applicant, applicant's representative, PTO personnel):

(1) *Angela M. Bertagna*.

(2) *Ian Lodovice (Applicant's representative)*.

(3) *Richard Roth (Applicant)*.

(4) *Belinda Lew (Applicant's representative)*.

Date of Interview: *15 September 2015*.

Type: ☒ Telephonic ☐ Video Conference
☐ Personal [copy given to: ☐ applicant ☐ applicant's representative]

Exhibit shown or demonstration conducted: ☐ Yes ☒ No.
If Yes, brief description: _____.

Issues Discussed ☐101 ☐112 ☐102 ☒103 ☐Others
(For each of the checked box(es) above, please describe below the issue and detailed description of the discussion)

Claim(s) discussed: *67 and 96-107*.

Identification of prior art discussed: *Wick (US 6,063,604), Kong (US 6,191,267), and Yao (US 20090092967), all of which are of record*.

Substance of Interview
(For each issue discussed, provide a detailed description and indicate if agreement was reached. Some topics may include: identification or clarification of a reference or a portion thereof, claim interpretation, proposed amendments, arguments of any applied references etc...)

We discussed the response filed on 7/27/15. The examiner stated that, in view of Applicant's arguments regarding Yao and also in view of the disclosure of Wick at column 25, second paragraph, claim 67 may be rejected under 103(a) as being unpatentable over Wick in view of Kong OR Wick in view of Kong and further in view of a new reference indicating that denaturing single-stranded RNA targets is not necessary in SDA. The examiner also stated that the new claims requiring short amplification times would not be rejected with Wick, Kong, and Yao, but that further search and consideration would be done. The examiner agreed to call Applicant's representative if an examiner's amendment could be made to place the case in condition for allowance..

Applicant recordation instructions: The formal written reply to the last Office action must include the substance of the interview. (See MPEP section 713.04). If a reply to the last Office action has already been filed, applicant is given a non-extendable period of the longer of one month or thirty days from this interview date, or the mailing date of this interview summary form, whichever is later, to file a statement of the substance of the interview

Examiner recordation instructions: Examiners must summarize the substance of any interview of record. A complete and proper recordation of the substance of an interview should include the items listed in MPEP 713.04 for complete and proper recordation including the identification of the general thrust of each argument or issue discussed, a general indication of any other pertinent matters discussed regarding patentability and the general results or outcome of the interview, to include an indication as to whether or not agreement was reached on the issues raised.

☐ Attachment

/Angela M. Bertagna/ Primary Examiner, Art Unit 1637	

Summary of Record of Interview Requirements

Manual of Patent Examining Procedure (MPEP), Section 713.04, Substance of Interview Must be Made of Record
A complete written statement as to the substance of any face-to-face, video conference, or telephone interview with regard to an application must be made of record in the application whether or not an agreement with the examiner was reached at the interview.

Title 37 Code of Federal Regulations (CFR) § 1.133 Interviews
Paragraph (b)

In every instance where reconsideration is requested in view of an interview with an examiner, a complete written statement of the reasons presented at the interview as warranting favorable action must be filed by the applicant. An interview does not remove the necessity for reply to Office action as specified in §§ 1.111, 1.135. (35 U.S.C. 132)

37 CFR §1.2 Business to be transacted in writing.
All business with the Patent or Trademark Office should be transacted in writing. The personal attendance of applicants or their attorneys or agents at the Patent and Trademark Office is unnecessary. The action of the Patent and Trademark Office will be based exclusively on the written record in the Office. No attention will be paid to any alleged oral promise, stipulation, or understanding in relation to which there is disagreement or doubt.

The action of the Patent and Trademark Office cannot be based exclusively on the written record in the Office if that record is itself incomplete through the failure to record the substance of interviews.

It is the responsibility of the applicant or the attorney or agent to make the substance of an interview of record in the application file, unless the examiner indicates he or she will do so. It is the examiner's responsibility to see that such a record is made and to correct material inaccuracies which bear directly on the question of patentability.

Examiners must complete an Interview Summary Form for each interview held where a matter of substance has been discussed during the interview by checking the appropriate boxes and filling in the blanks. Discussions regarding only procedural matters, directed solely to restriction requirements for which interview recordation is otherwise provided for in Section 812.01 of the Manual of Patent Examining Procedure, or pointing out typographical errors or unreadable script in Office actions or the like, are excluded from the interview recordation procedures below. Where the substance of an interview is completely recorded in an Examiners Amendment, no separate Interview Summary Record is required.

The Interview Summary Form shall be given an appropriate Paper No., placed in the right hand portion of the file, and listed on the "Contents" section of the file wrapper. In a personal interview, a duplicate of the Form is given to the applicant (or attorney or agent) at the conclusion of the interview. In the case of a telephone or video-conference interview, the copy is mailed to the applicant's correspondence address either with or prior to the next official communication. If additional correspondence from the examiner is not likely before an allowance or if other circumstances dictate, the Form should be mailed promptly after the interview rather than with the next official communication.

The Form provides for recordation of the following information:
– Application Number (Series Code and Serial Number)
– Name of applicant
– Name of examiner
– Date of interview
– Type of interview (telephonic, video-conference, or personal)
– Name of participant(s) (applicant, attorney or agent, examiner, other PTO personnel, etc.)
– An indication whether or not an exhibit was shown or a demonstration conducted
– An identification of the specific prior art discussed
– An indication whether an agreement was reached and if so, a description of the general nature of the agreement (may be by attachment of a copy of amendments or claims agreed as being allowable). Note: Agreement as to allowability is tentative and does not restrict further action by the examiner to the contrary.
– The signature of the examiner who conducted the interview (if Form is not an attachment to a signed Office action)

It is desirable that the examiner orally remind the applicant of his or her obligation to record the substance of the interview of each case. It should be noted, however, that the Interview Summary Form will not normally be considered a complete and proper recordation of the interview unless it includes, or is supplemented by the applicant or the examiner to include, all of the applicable items required below concerning the substance of the interview.

A complete and proper recordation of the substance of any interview should include at least the following applicable items:
1) A brief description of the nature of any exhibit shown or any demonstration conducted,
2) an identification of the claims discussed,
3) an identification of the specific prior art discussed,
4) an identification of the principal proposed amendments of a substantive nature discussed, unless these are already described on the Interview Summary Form completed by the Examiner,
5) a brief identification of the general thrust of the principal arguments presented to the examiner,
   (The identification of arguments need not be lengthy or elaborate. A verbatim or highly detailed description of the arguments is not required. The identification of the arguments is sufficient if the general nature or thrust of the principal arguments made to the examiner can be understood in the context of the application file. Of course, the applicant may desire to emphasize and fully describe those arguments which he or she feels were or might be persuasive to the examiner.)
6) a general indication of any other pertinent matters discussed, and
7) if appropriate, the general results or outcome of the interview unless already described in the Interview Summary Form completed by the examiner.

Examiners are expected to carefully review the applicant's record of the substance of an interview. If the record is not complete and accurate, the examiner will give the applicant an extendable one month time period to correct the record.

Examiner to Check for Accuracy

If the claims are allowable for other reasons of record, the examiner should send a letter setting forth the examiner's version of the statement attributed to him or her. If the record is complete and accurate, the examiner should place the indication, "Interview Record OK" on the paper recording the substance of the interview along with the date and the examiner's initials.

PTO/SB/30 (07-09)
Approved for use through 07/31/2012. OMB 0651-0031
U.S. Patent and Trademark Office; U.S. DEPARTMENT OF COMMERCE
Under the Paperwork Reduction Act of 1995, no persons are required to respond to a collection of information unless it contains a valid OMB control number.

Request for Continued Examination (RCE) Transmittal

Address to:
Mail Stop RCE
Commissioner for Patents
P.O. Box 1450
Alexandria, VA 22313-1450

Application Number	14/067,620
Filing Date	October 30, 2013
First Named Inventor	Brian K. Maples
Art Unit	1637
Examiner Name	Olayinka A. Oyeyemi
Attorney Docket Number	30171-0025002

This is a Request for Continued Examination (RCE) under 37 CFR 1 .114 of the above-identified application.
Request for Continued Examination (RCE) practice under 37 CFR 1.114 does not apply to any utility or plant application filed prior to June 8, 1995, or to any design application. See Instruction Sheet for RCEs (not to be submitted to the USPTO) on page 2.

1. Submission required under 37 CFR 1.114 Note: If the RCE is proper, any previously filed unentered amendments and amendments enclosed with the RCE will be entered in the order in which they were filed unless applicant instructs otherwise. If applicant does not wish to have any previously filed unentered amendment(s) entered, applicant must request non-entry of such amendment(s).
   - a. ☐ Previously submitted. If a final Office action is outstanding, any amendments filed after the final Office action may be considered as a submission even if this box is not checked.
     - i. ☐ Consider the arguments in the Appeal Brief or Reply Brief previously filed on ____
     - ii. ☐ Other ____
   - b. ☒ Enclosed
     - i. ☒ Amendment/Reply
     - ii. ☐ Affidavit(s)/ Declaration(s)
     - iii. ☐ Information Disclosure Statement (IDS)
     - iv. ☐ Other ____

2. Miscellaneous
   Suspension of action on the above-identified application is requested under 37 CFR 1.103(c) for a
   - a. ☐ period of ____ months. (Period of suspension shall not exceed 3 months; Fee under 37 CFR 1.17(i) required)
   - b. ☐ Other ____

3. Fees The RCE fee under 37 CFR 1.17(e) is required by 37 CFR 1.114 when the RCE is filed.
   The Director is hereby authorized to charge the following fees any underpayment of fees or credit any overpayments to
   - a. ☒ Deposit Account No. 06-1050 .
     - i. ☒ RCE fee required under 37 CFR 1.17(e)
     - ii ☒ Extension of time fee (37 CFR 1.136 and 1.17)
     - iii ☒ Other any deficiencies
   - b. ☐ Check in the amount of $ ____ enclosed
   - c. ☐ Payment by credit card (Form PTO-2038 enclosed)

WARNING: Information on this form may become public. Credit card information should not be included on this form. Provide credit card information and authorization on PTO-2038.

SIGNATURE OF APPLICANT, ATTORNEY, OR AGENT REQUIRED

Signature	/Ian J.S. Lodovice, Reg. No. 59,749/	Date	July 27, 2015
Name (Print/Type)	Ian J. Lodovice	Registration No.	59,749

CERTIFICATE OF MAILING OR TRANSMISSION

I hereby certify that this correspondence is being deposited with the United States Postal Service with sufficient postage as first class mail in an envelope addressed to: Mail Stop RCE, Commissioner for Patents, P. O. Box 1450, Alexandria, VA 22313-1450 or facsimile transmitted to the U.S. Patent and Trademark Office on the date shown below.

Signature			
Name (Print/Type)		Date	

This collection of information is required by 37 CFR 1.114. The information is required to obtain or retain a benefit by the public which is to file (and by the USPTO to process) an application. Confidentiality is governed by 35 U.S.C. 122 and 37 CFR 1.11 and 1.14. This collection is estimated to take 12 minutes to complete, including gathering, preparing, and submitting the completed application form to the USPTO. Time will vary depending upon the individual case. Any comments on the amount of time you require to complete this form and/or suggestions for reducing this burden, should be sent to the Chief Information Officer, U.S. Patent and Trademark Office, U.S. Department of Commerce, P.O. Box 1450, Alexandria, VA 22313-1450. DO NOT SEND FEES OR COMPLETED FORMS TO THIS ADDRESS. **SEND TO: Mail Stop RCE, Commissioner for Patents, P.O. Box 1450, Alexandria, VA 22313-1450.**
If you need assistance in completing the form, call 1-800-PTO-9199 and select option 2.

American LegalNet, Inc.
www.FormsWorkFlow.com

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

First Named Inventor	:	Brian K. Maples	Art Unit	:	1637
Serial No.	:	14/067,620	Examiner	:	Olayinka A. Oyeyemi
Filed	:	October 30, 2013	Conf. No.	:	4288

Title : NICKING AND EXTENSION AMPLIFICATION REACTION FOR THE EXPONENTIAL AMPLIFICATION OF NUCLEIC ACIDS

MAIL STOP AF
Commissioner for Patents
P.O. Box 1450
Alexandria, VA 22313-1450

AMENDMENT AND REPLY TO ACTION OF FEBRUARY 27, 2015 AND TO ADVISORY ACTION OF JUNE 11, 2015

Please consider the following amendment and reply.

The present amendment is accompanied by a Request for Continued Examination (RCE).

List of claims (replacing prior versions).

1. - 66. (Canceled)

67. (Currently Amended) A method of amplifying a target polynucleotide sequence of a target nucleic acid present in a sample obtained from an animal, the method comprising:

(a) preparing, without first subjecting the target nucleic acid to a thermal denaturation step associated with amplification of the target polynucleotide sequence, a mixture comprising:

(i) the target nucleic acid comprising the target polynucleotide sequence,

(ii) a polymerase,

(iii) a nicking enzyme,

(iv) a first oligonucleotide comprising a nicking site and a nicking enzyme binding site, and

(v) a second oligonucleotide comprising a nicking site and a nicking enzyme binding site, and

(b) subjecting the mixture to essentially isothermal conditions to amplify the target polynucleotide sequence.

68. (Previously Presented) The method of claim 67, wherein the target polynucleotide sequence is amplified from steps comprising:

(a) forming a first duplex comprising the target polynucleotide sequence and the first oligonucleotide;

(b) extending, using the polymerase, the first oligonucleotide along the target polynucleotide sequence to form an extended first oligonucleotide comprising a sequence complementary to the second oligonucleotide;

(c) forming a second duplex comprising the second oligonucleotide and the extended first oligonucleotide;

(d) extending, using the polymerase, the second oligonucleotide along the extended first oligonucleotide to form a third duplex comprising an extended second oligonucleotide comprising a sequence complementary to the first oligonucleotide and a first double-stranded nicking enzyme binding site;

(e) nicking, with the nicking enzyme, the first nicking site on the third duplex to produce a fourth duplex comprising the extended second oligonucleotide and a fragment of the extended first oligonucleotide; and

(f) extending, using the polymerase, the fragment of the extended first oligonucleotide along the extended second oligonucleotide of the fourth duplex to produce a double-stranded nucleic acid product and a second double-stranded nicking enzyme binding site.

69. (Previously Presented) The method of claim 68, wherein the double-stranded nucleic acid product comprises:

i) a first strand and a second strand, wherein the first strand comprises a first polynucleotide sequence corresponding to the target polynucleotide sequence and the second strand comprises a second polynucleotide sequence complementary to the target polynucleotide sequence, and

ii) first and second double-stranded nicking sites spaced apart by the target polynucleotide sequence.

70. (Previously Presented) The method of claim 68, further comprising the steps of:

a) nicking, using the nicking enzyme, the first nicking site of the double-stranded nucleic acid product to produce a fifth duplex comprising a first polynucleotide sequence corresponding to the target polynucleotide sequence and a fragment of the first oligonucleotide, and nicking, using the nicking enzyme, the second nicking site of the double-stranded nucleic acid product to produce a sixth duplex comprising a second polynucleotide sequence complementary to the target polynucleotide sequence and a fragment of the second oligonucleotide;

b) extending, using the polymerase, the fragment of the first oligonucleotide along the first polynucleotide sequence of the fifth duplex to produce a first double stranded product comprising a copy of the nicking site and a copy of the first polynucleotide sequence and

extending, using the polymerase, the fragment of the second oligonucleotide along the second polynucleotide sequence of the sixth duplex to produce a second double stranded product comprising a copy of the nicking site and a copy of the second polynucleotide sequence; and

c) nicking, using the nicking enzyme, the copy of the nicking site of the first double stranded product to release a copy of the first polynucleotide sequence and nicking, using the nicking enzyme, the copy of the nicking site of the second double stranded product to release a copy of the second polynucleotide sequence.

71. (Previously Presented) The method of claim 67, wherein the animal is a human.

72. (Previously Presented) The method of claim 67, wherein the target nucleic acid is obtained from an animal pathogen.

73. (Previously Presented) The method of claim 72, wherein the animal pathogen is a single-stranded DNA virus, double-stranded DNA virus, or single-stranded RNA virus.

74. (Withdrawn) The method of claim 72, wherein the animal pathogen is a bacterium.

75. (Withdrawn) The method of claim 72, wherein the animal pathogen contains spores and the target polynucleotide is amplified from the spores without the need for lysis of the spores.

76. (Previously Presented) The method of claim 67, wherein the sample obtained from an animal is obtained from the blood, bone marrow, mucus, lymph, hard tissues, biopsies, sputum, saliva, tears, faeces or urine of the animal.

77. (Previously Presented) The method of claim 76, wherein the sample obtained from an animal is obtained from the mucus, sputum, or saliva of the animal.

78. (Withdrawn) The method of claim 67, wherein the target nucleic acid is double-stranded DNA.

79. (Withdrawn) The method of claim 67, wherein the target nucleic acid is single-stranded DNA.

80. (Previously Presented) The method of claim 67, wherein the target nucleic acid is RNA.

81. (Withdrawn) The method of claim 67, wherein the target nucleic acid is selected from the group consisting of genomic DNA, plasmid DNA, viral DNA, mitochondrial DNA, cDNA, synthetic double-stranded DNA and synthetic single-stranded DNA.

82. (Withdrawn) The method of claim 81, wherein the target nucleic acid is genomic DNA.

83. (Previously Presented) The method of claim 67, wherein the target nucleic acid is viral DNA or viral RNA.

84. (Canceled)

85. (Previously Presented) The method of claim 67, wherein the nicking enzyme is Nt.BstNBI.

86. (Previously Presented) The method of claim 67, wherein the nicking enzyme does not nick within the target polynucleotide sequence.

87. (Previously Presented) The method of claim 67, wherein amplification of the target polynucleotide sequence is performed without the use of temperature cycling.

88. (Previously Presented) The method of claim 67, wherein amplification of the target polynucleotide sequence is performed at about 55°C-59°C.

89. (Canceled)

90. (Previously Presented) The method of claim 68, which is performed at a temperature higher than the melting temperature of the first oligonucleotide/target polynucleotide sequence complex.

91. (Previously Presented) The method of claim 67, further comprising detecting amplification product.

92. (Previously Presented) The method of claim 91, wherein the amplification product is detected by a detection method selected from the group consisting of gel electrophoresis, mass spectrometry, fluorescence, intercalating dye detection, fluorescence resonance energy transfer (FRET), molecular beacon detection, surface capture, capillary electrophoresis, incorporation of labeled nucleotides to allow detection by capture, fluorescence polarization, and lateral flow capture, or a combination thereof.

93. (Canceled)

94. (Canceled)

95. (Withdrawn, Currently Amended) A method of amplifying a target polynucleotide sequence of genomic DNA present in a sample obtained from an animal, the method comprising:

(a) preparing, without first subjecting the genomic DNA to a thermal denaturation step associated with amplification of the target polynucleotide sequence, a mixture comprising:

(i) the genomic DNA comprising the target polynucleotide sequence,

(ii) a polymerase,

(iii) a nicking enzyme,

(iv) a first oligonucleotide comprising a nicking site and a nicking enzyme binding site, and

(v) a second oligonucleotide comprising a nicking site and a nicking enzyme binding site, and

b) subjecting the mixture to essentially isothermal conditions to amplify the target polynucleotide sequence.

96. (New) The method of claim 67, wherein the target polynucleotide sequence is amplified about 1E+8-fold.

97. (New) The method of claim 67, wherein the target polynucleotide sequence is amplified about 3E+9-fold.

98. (New) The method of claim 67, wherein the target polynucleotide sequence is amplified about 7E+10-fold.

99. (New) The method of claim 67, wherein amplification of the target polynucleotide sequence is performed at a constant temperature for about 1 to 12 minutes

100. (New) The method of claim 67, wherein amplification of the target polynucleotide sequence is performed at a constant temperature for about 1 to 10 minutes.

101. (New) The method of claim 67, wherein amplification of the target polynucleotide sequence is performed at a constant temperature for about 1 to 8 minutes.

102. (New) The method of claim 67, wherein amplification of the target polynucleotide sequence is performed at a constant temperature for about 1 to 5 minutes.

103. (New) The method of claim 67, wherein amplification of the target polynucleotide sequence is performed at a constant temperature for about 1 to 2.5 minutes.

104. (New) The method of claim 67, wherein amplification of the target polynucleotide sequence is performed at a constant temperature for about 2.5 to 5 minutes.

105. (New) The method of claim 67, wherein amplification of the target polynucleotide sequence is performed at a constant temperature for about 2.5 to 8 minutes.

106. (New) The method of claim 67, wherein amplification of the target polynucleotide sequence is performed at a constant temperature for about 2.5 to 10 minutes.

107. (New) A method of amplifying a target polynucleotide sequence of a target nucleic acid present in a sample obtained from an animal, the method comprising:

(a) preparing, without first subjecting the target nucleic acid to a denaturation step associated with amplification of the target polynucleotide sequence, a mixture comprising:

(i) the target nucleic acid comprising the target polynucleotide sequence,

(ii) a polymerase,

(iii) a nicking enzyme,

(iv) a first oligonucleotide comprising a nicking site and a nicking enzyme binding site, and

(v) a second oligonucleotide comprising a nicking site and a nicking enzyme binding site, and

(b) subjecting the mixture to essentially isothermal conditions to amplify the target polynucleotide sequence,

wherein the target polynucleotide sequence is amplified about 1E+8-fold in about 1 to 12 minutes.

REMARKS

Upon entry of the above amendment, claims 67-83, 85-88, 90-92 and 95-107 will be pending. Claims 1-66, 84 and 93-94 were previously canceled, and claim 89 is newly canceled. Claims 74-75, 78-79, 81-82 and 95 were previously withdrawn, so are not currently under examination. Claims 67 (and withdrawn claim 95) have been amended and new claims 96-107 have been added. Applicants note that the amendments submitted by Applicants in the AMENDMENT AND REPLY TO FINAL ACTION OF FEBRURY 27, 2015 filed May 27, 2015 have not been entered (see Advisory Action dated June, 11, 2015) and the amendments submitted herewith reflect this.

Most of the amendments are simply to clarify scope and are supported throughout the specification. For example, the amendment to claims 67 and 95 find support at page 4, lines 16-30, and in Example 11. Claims 96-106 derive support from the specification at page 28, lines 4-5; and Examples 10-11. Claim 107 derives support from claim 67; and Examples 10-11.

No new matter has been introduced by these amendments. Reconsideration and allowance of the claims are respectfully requested in view of the above amendments and the following remarks.

Interview Summary

Applicant thanks Examiner Bertagna for the courtesy of a telephonic interview with Applicant's representatives Ian Lodovice, Rich Roth, and Belinda Lew on June 12, 2015. During the interview, the participants discussed the new matter rejection under 35 U.S.C. §§ 112, first paragraph and amendments for placing claims in conditions for allowance. The amendments to claims presented above are substantially as discussed by the participants during the interview. Applicant acknowledges and thanks Examiner Bertagna for the Applicant-Initiated Interview Summary dated June 17, 2015.

Claim Rejection Under 35 USC § 112, First Paragraph (New Matter)

Claims 67-73, 76, 77, 80, 83 and 85-92 stand rejected under the first paragraph of 35 U.S.C. § 112 for allegedly failing to comply with the written description requirement. Specifically, the Office Action contends that Applicant's disclosure does not sufficiently provide support for "any type of denaturation step associated with amplification during the mixture preparation step." This contention is respectfully traversed for the reasons outlined in Applicants response filed on May 27, 2015. However, without conceding the appropriateness of the present rejection, and solely in the interest of advancing prosecution, claim 67 has been amended to recite "without first subjecting the target nucleic acid to a thermal denaturation step associated with amplification of the target polynucleotide sequence." In view of the foregoing, Applicant respectfully submits that ordinarily skilled artisans would reasonably conclude that Applicant has adequately described the claimed methods. Reconsideration and withdrawal of the rejection of under 35 U.S.C. 112, first paragraph is requested.

Claim Rejection Under 35 USC § 103

Claims 67-73, 76, 77, 80, 83, and 85-92 stand rejected under pre-AIA 35 U.S.C. 103(a) as allegedly being unpatentable over Wick et al. (US 6,063,604)("Wick") in view of Kong et al. (US 6,191,267)("Kong") and further in view of Yao et al. (US 2009/0092967)("Yao"). As none of the references cited by the Office, considered along or in combination disclose each and every feature of amended claim 67, Applicant respectfully traverses.

As described in the present application, Applicants have discovered novel methods of amplifying target sequences that rely on nicking and extension reactions which amplify sequences in less time that prior amplification reactions, such as, for example strand displacement amplification (SDA). The amplification reactions described herein use only two templates (e.g., a first oligonucleotide and a second oligonucleotide, as claimed), one or more nicking enzymes and a polymerase to amplify a target under isothermal conditions. The claimed methods provide significant advantages over prior amplification reactions, such as those described in Wick and Kong, including the lack of a requirement for an initial thermal denaturation step.

The establishment of a *prima facie* case of obviousness requires three criteria: 1) a teaching, motivation, or suggestion must be shown by the Office to combine the cited references; 2) all claim limitations must be taught or suggested by the references; and 3) a reasonable expectation of success on the part of a skilled practitioner in making the combination of the references. In the present case, the Office has not provided a teaching, motivation, or suggestion to combine the cited references; has not cited references that teach all of the claim limitations; and has not provided a reasonable expectation of success in making the combination. Thus, a prima facie case of obviousness cannot be established by the instant combination of the references.

As an initial matter, the Office acknowledges that neither Wick nor Kong teach a method of amplifying a target polynucleotide sequence of a target nucleic acid in a sample, without first subjecting the target nucleic acid to a denaturation step associated with amplification of the target polynucleotide sequence, as required by the present claims. However, in a new §103(a) rejection, the Office cites Yao for teaching the omission of an initial pre-amplification denaturation step, arguing that Yao provides the requisite motivation and reasonable expectation of success for omitting the initial denaturation step when practicing the method allegedly suggested by the teachings of Wick and Kong.

Applicant disagrees that there is any motivation to combine or any reasonable expectation of success for the Office's proposed combination of Wick, Kong and Yao. Yao teaches the elimination of thermal denaturation solely in the context of generating single-stranded target nucleic acid from a double-stranded target molecule (e.g., as illustrated in Figure 1A of Yao), for subsequent SDA amplification of the single-stranded target nucleic acid (e.g., as illustrated in Figure 1B of Yao): "[i]n contrast to traditional "isothermal SDA" which require at least one thermal denaturation step at about 95°C to generate single stranded DNA (ssDNA), the isothermal SDA of the invention generates ssDNA isothermally by polymerase strand displacement and, accordingly eliminates the thermal denaturation step." Yao, paragraph [0029] as cited by the examiner, emphasis added. However, the primary Wick reference is cited by the Office for teaching target nucleic acid that is already single-stranded, e.g., single-stranded viral RNA. To the extent Wick shows that a denaturation step is required to prepare target nucleic acid that is originally single-stranded (e.g. RNA templates described in Wick's Examples) for

subsequent amplification, this denaturation step is clearly not in the context of generating single-stranded target from an originally double-stranded molecule. Yao simply fails to provide any motivation to omit the prior denaturation step as taught by Wick and is not even relevant to amplification of target nucleic acid that is already single-stranded. For at least these reasons, the skilled artisan, if looking to modify Wick to arrive at the present claims, would not have even looked to Yao. Yao fails to provide any motivation to combine its teachings with Wick and Kong, and further fails to provide any reasonable expectation that the suggested method of Wick and Kong would work, if the pre-amplification denaturation step as taught by Wick was omitted for originally single-stranded nucleic acid targets.

Further evidence that the Applicant's claimed invention is novel and unexpected is the recognition by professional health organizations such as the Centers for Disease Control (CDC) of Applicant's "Alere i" product[1] as the first one of a "new type" of molecular diagnostic test based on isothermal nucleic acid amplification technology that the CDC has named "rapid molecular assay" due to its unique ability to provide "high sensitivity and yield[s] results in 15 minutes or less" (e.g., Alere i NAT Flu A/B).[2] Alere i is a commercially available, instrument-based, isothermal system for the qualitative detection of infectious diseases, which embodies the technology and invention disclosed and presently claimed. It is not surprising that the rapid molecular diagnostic test embodied in Alere i is recognized by organizations such as the CDC as a "new type" of molecular diagnostic test. Prior to the invention disclosed and presently claimed, other isothermal nucleic amplification technologies have been developed, but none were able to perform, or were expected to perform, the methods as presently claimed.

For at least these reasons, independent claim 67 is patentable in view of the combination of Wick, Kong and Yao. Because independent claim 67 is patentable over Wick, Kong and Yao, dependent claims 68-73, 76, 77, 80, 83, and 85-88 and 90-92 are also patentable over Wick,

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[1] See http://www.alere.com/us/en/product-details/alere-i.html; http://www.alere.com/us/en/product-details/alere-i-influenza-ab.html; and http://www.alere.com/us/en/product-details/alere-i-strep-a.html.

[2] **Exhibit 1** (Guidance for Clinicians on the Use of RT-PCR and Other Molecular Assays For Diagnosis of Influenza Virus Infection", see http://www.cdc.gov/flu/professionals/diagnosis/molecular-assays.htm); and **Exhibit 2** (FDA grants first CLIA waiver for nucleic acid-based flu diagnostic, "The Alere i Influenza A & B test uses a nasal swab sample from a patient with signs and symptoms of flu infection. The test provides results in as little as 15 minutes and may be performed in the presence of the patient", emphasis added, see http://www.fda.gov/NewsEvents/Newsroom/PressAnnouncements/ucm429127.htm).

Kong and Yao. For at least these reasons, Applicant requests reconsideration and withdrawal of this rejection.

Double Patenting

Claims 67-73, 76, 77, 80, 83, and 85-92 stand provisionally rejected on the ground of nonstatutory double patenting as being allegedly unpatentable over claims 67-69, 74-76, 79, 80, 83, 85, and 87-94 of copending Application No. 14/067,623.

Claims 67-73, 76, 77, 80, 83, and 85-92 stand provisionally rejected on the ground of nonstatutory double patenting as being allegedly unpatentable over claims 1-9, 12, 14-17, 19-41, and 44- 46 of copending Application No. 12/173,020.

Claims 67-73, 76, 77, 80, 83, 86-88, and 90-92 stand provisionally rejected on the ground of nonstatutory double patenting as being allegedly unpatentable over claims 125-130 of copending Application No. 11/778,018 in view of Wick.

Claims 85 and 89 are provisionally rejected stand provisionally rejected on the ground of nonstatutory double patenting as being allegedly unpatentable over claims 125-130 of copending Application No. 11/778,018 in view of Wick and further in view of Kong .

Applicant does not concede that the rejections above are appropriate. Further, as the applications cited above are all currently pending, Applicant requests that the rejections be held in abeyance pending the identification of allowable subject matter.

CONCLUSION

In light of the arguments made herein, Applicant submit that the pending claims are patentable and request early and favorable action thereon. If any issues remain, the Examiner is asked to telephone the Applicants' representative Ian Lodovice at 617-956-5972 to arrange a time for an interview.

Applicant does not concede any positions of the Office that are not expressly addressed above, nor does Applicant concede that there are not other good reasons for patentability of the presented claims or other claims.

This response is being filed with a Request for Continued Examination and the required fees which are being paid concurrently herewith on the Electronic Filing System (EFS) by way of Deposit Account authorization. The fees for the Two-Month extension of time in the amount of $600.00 are also being paid with this reply on the Electronic Filing System. Apply those fees and any other necessary charges or credits to Deposit Account 06-1050, referencing the above attorney docket number.

Respectfully submitted,

Date: July 27, 2015__________________

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EXHIBIT 1

(http://www.cdc.gov/flu/professionals/diagnosis/molecular-assays.htm)

Centers for Disease Control and Prevention
CDC 24/7: Saving Lives. Protecting People™

Guidance for Clinicians on the Use of RT-PCR and Other Molecular Assays for Diagnosis of Influenza Virus Infection

Background

Tests for influenza include molecular assays, rapid influenza diagnostic tests, viral culture or serology. This guidance focuses upon molecular assays for influenza as they are increasingly being used in clinical settings (1). Reverse Transcription-Polymerase Chain Reaction (RT-PCR) and other molecular assays can identify the presence of influenza viral RNA in respiratory specimens. (See Table 1 below.) Some molecular assays are able to detect and discriminate between infections with influenza A and B viruses; other tests can identify specific influenza A virus subtypes [A(H1N1)pdm09, seasonal A (H1N1), or seasonal A (H3N2)]. These assays can yield results in 3-8 hours. Notably, the detection of influenza viral RNA by these assays does not always indicate detection of viable virus or on-going influenza viral replication. It is important to note that not all assays have been cleared by the FDA for diagnostic use. FDA-cleared assays are listed in Table 1.

[1] One FDA-cleared rapid molecular assay is available in the United States. This assay has high sensitivity and yields results in 15 minutes.

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- Detecting Novel Influenza A Cases
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- References
- Table 1. FDA-cleared RT-PCR Assays and Other Molecular Assays for Influenza Viruses (PDF Available)

Use in Clinical Decision Making

- Influenza testing is not needed for all patients with signs and symptoms of influenza to make antiviral treatment decisions (See Figure 1, Figure 2). Once influenza activity has been identified in the community or geographic area, a clinical diagnosis of influenza can be made for outpatients with signs and symptoms consistent with suspected influenza, especially during periods of peak influenza activity in the community. For most outpatients and emergency room patients, molecular assays are not available and results will not be available in a timely manner to inform clinical decision-making.
  - Molecular testing is not needed on all patients with suspected influenza, but is most appropriate for hospitalized patients if a positive test would result in a change in clinical management.
  - Clinicians should be aware of the approved clinical specimens for the molecular assay being used (see Table 1. FDA-cleared RT-PCR Assays and Other Molecular Assays for Influenza Viruses (202 KB, 3 pages)).
- If treatment is clinically indicated, antiviral treatment should NOT be withheld from patients with suspected influenza while awaiting testing results during periods of peak influenza activity in the community when the likelihood of influenza is high. More information about antiviral treatment of influenza is available at Antiviral Drugs, Information for Health Care Professionals.

- Since results from molecular assays are usually not available when initial therapy decisions must be made, antiviral treatment should be started as soon as possible because the greatest benefit is when treatment is initiated as close to illness onset as possible, especially for patients at high risk of serious outcomes.

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Influenza Testing of Hospitalized Patients

- Hospitalized patients with suspected influenza without lower respiratory tract disease should have upper respiratory tract specimens collected for influenza testing. More information about antiviral treatment of influenza is available at Antiviral Drugs, Information for Health Care Professionals.
- Collection of lower respiratory tract specimens from hospitalized patients with suspected influenza and pneumonia can be considered for influenza testing by RT-PCR if influenza testing of upper respiratory tract specimens is negative and if positive testing would result in a change in clinical management. Hospitalized patients with suspected influenza and respiratory failure on mechanical ventilation can have an endotracheal aspirate specimen collected for influenza testing by RT-PCR if a laboratory diagnosis of influenza has not been determined. Bronchoalveolar lavage fluid, if collected for other diagnostic purposes, can also be tested by RT-PCR for influenza viruses. Currently, only the CDC RT-PCR assay is FDA-cleared for lower respiratory tract specimens; this test is available only at qualified public health laboratories (see Table 1. FDA-cleared RT-PCR Assays and Other Molecular Assays for Influenza Viruses [202 KB, 3 pages]). Clinicians may elect to order other FDA-cleared assays for off-label use in evaluating lower respiratory tract specimens. Performance of these assays for these specimens has not been evaluated by FDA; however, these assays may be more readily accessible at some institutions.

Use in Detecting Institutional Influenza Outbreaks

- Molecular assays such as RT-PCR are particularly useful to identify influenza virus infection as a cause of respiratory outbreaks in institutions (e.g., nursing homes, chronic care facilities, and hospitals).
- Positive results from one or more ill persons with suspected influenza can support decisions to promptly implement prevention and control measures for influenza outbreaks. Clinicians should be aware of requirements from their public health authorities regarding notification of any suspected or confirmed institutional influenza outbreaks, and when respiratory specimens should be collected from ill persons and sent to a public health laboratory for laboratory confirmation of influenza.

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Use in Detecting Novel Influenza A Cases

- Molecular assays, such as RT-PCR, are designed to accurately identify influenza A and B viral RNA by using conserved gene targets. Some assays will detect influenza A or B viruses but will not determine the influenza A virus subtype, and thus will not be able to indicate if the infection is due to a novel influenza A virus.
- Some FDA-cleared devices can not only detect influenza A or B, but also can identify influenza A hemagglutinin genes, allowing for determination of some or all of the seasonal influenza A virus subtypes [i.e., A(H1N1)pdm09, seasonal A (H1N1), or seasonal A (H3N2)]. These assays will not only identify the currently circulating influenza A strains, but also may identify viruses that are detected as influenza A for which no subtype could be identified. These "unsubtypables" may represent novel influenza A virus infections.
- Clinicians and laboratorians using molecular assays that are capable of detecting all currently circulating influenza A virus subtypes [i.e., A(H1N1)pdm09, seasonal A (H1N1), or seasonal A (H3N2)], and who identify an

"unsubtypable" result (i.e., influenza A with no subtype detected), should contact their state or local public health laboratory immediately for additional testing to determine if the infection is due to a novel influenza A virus.

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Factors Influencing Results of Molecular Assays

Many factors can influence influenza testing results. Influenza viral shedding in the upper respiratory tract generally declines substantially after 4 days in patients with uncomplicated influenza. Patients with lower respiratory tract disease may have prolonged influenza viral replication in the lower respiratory tract. Molecular tests can detect influenza viral RNA (positive results) for a longer duration than other influenza testing (e.g. antigen testing - immunofluorescence or rapid influenza diagnostic tests). Although RT-PCR is the most sensitive influenza test and is highly specific, negative results can occur in persons with influenza for multiple reasons so negative RT-PCR results may not always exclude a diagnosis of influenza. If clinical suspicion of influenza is high, antiviral treatment should continue in patients with severe illness or at high risk for complications while additional respiratory specimens and influenza testing is performed.

Factors that can influence influenza testing results are:

- Time from illness onset to collection of respiratory specimens for testing
  - Respiratory specimens should be collected as early as possible (ideally less than 48-72 hours after illness onset when viral shedding is highest) in persons without lower respiratory tract disease and tested as soon as possible.
- Source of respiratory specimens tested and specimen handling
  - The best upper respiratory tract specimens to detect influenza viral RNA by RT-PCR and other molecular assays are nasopharyngeal swabs, washes or aspirates; other acceptable specimens are a nasal and/or throat swab. A swab with a wood shaft should not be used for respiratory specimen collection because it may interfere with RT-PCR and other molecular assays. Clinicians should be aware of the approved clinical specimens for the molecular assay being used and what type of swabs are recommended for use with the assay as included in the manufacturer's instructions included in the assay.
  - Hospitalized patients with lower respiratory tract disease may have prolonged lower respiratory tract influenza viral replication compared to the upper respiratory tract. In patients with lower respiratory tract disease, lower respiratory tract specimens should be collected and tested if influenza is clinically suspected and testing of upper respiratory tract specimens is negative. For critically ill patients with suspected influenza, even when testing by RT-PCR or other molecular assays is negative, consideration should be given to collecting additional respiratory specimens from multiple sites, especially lower respiratory tract (endotracheal aspirate, or bronchoalveolar lavage – if clinically indicated for other diagnostic purposes) and re-tested for influenza viruses by RT-PCR or other molecular assays. Antiviral treatment should be continued in such patients pending additional influenza testing.
  - If testing is delayed or is done at a facility other than where the patient is hospitalized, specimens should be placed in sterile viral transport media, consistent with test specifications, and refrigerated until transported to the laboratory for testing as soon as possible. Freezing and thawing should be avoided or minimized to avoid degradation of influenza viruses if viral culture will be performed.
  - Manufacturer's instructions, including acceptable specimens, handling, and storage and processing, should be followed to achieve optimum test performance. Deviations from recommended procedures may result in false negative results.

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Interpretation of Testing Results

Sensitivities and specificities of RT-PCR and other molecular assays that have been cleared by the FDA for diagnostic use are high compared to other FDA-cleared assays which use different methods. However, even with RT-PCR, false negative results can occur due to improper or poor clinical specimen collection or from poor handling of a specimen after collection and before testing. A negative result can also occur by testing a specimen that was collected when the patient is no longer shedding detectable influenza virus. False positive results, although rare, can occur (e.g., due to lab contamination or other factors).

- Negative result
  - A negative result means that there is no evidence of influenza viral RNA in the specimen tested. For hospitalized patients, especially for patients with lower respiratory tract disease, if no other etiology is identified and influenza is still clinically suspected, additional specimens should be collected and tested, and antiviral treatment should be initiated or continued.
- Positive result
  - A positive result indicates detection of influenza viral RNA, confirming influenza virus infection, but does not necessarily mean viable virus is present or that the patient is contagious.
  - A positive result in a person who recently received intranasal administration of live attenuated influenza virus vaccine (LAIV) may indicate detection of vaccine virus. LAIV contains influenza virus strains that undergo viral replication in respiratory tissues of lower temperature (e.g. nasal passages) than internal body temperature. Since the nasal passages are infected with live influenza virus vaccine strains during LAIV administration, sampling the nasal passages within a few days after LAIV vaccination can yield positive influenza testing results. It may be possible to detect LAIV vaccine strains up to 7 days after vaccination, and in rare situations, for longer periods.
  - Influenza molecular assay interpretation will depend on the individual test that is performed. For example, a negative result from an influenza molecular assay that only detects influenza A virus and the A(H1N1)pdm09 subtype does not preclude infection with influenza B virus. Clinicians can consult for detailed descriptions of each FDA-cleared test and what the result may or may not signify.

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Advantages/Disadvantages of Molecular Assays

Advantages:

- Molecular assays are more sensitive and specific for detecting influenza viruses than other influenza tests (e.g., rapid influenza diagnostic tests, immunofluorescence, and viral culture)
- The likelihood of a false positive or false negative result is low and therefore, the interpretation of the result is less impacted by the level of influenza activity in the community
- Some, but not all molecular assays can distinguish between specific influenza A virus subtypes

Disadvantages:

- Results of RT-PCR and other molecular assays may not be available in a clinically relevant time frame to inform clinical management decisions.
- RT-PCR and other molecular assays are generally not available for outpatient or emergency room settings. For hospitalized patients, these assays are not always available on-site.

  - Respiratory specimens may need to be sent to a state public health laboratory or commercial laboratory for RT-PCR. Therefore, although the test can yield results in 3-8 hours, the actual time to receive results may be substantially longer.
- Most FDA-cleared molecular assays are not approved to test lower respiratory tract specimens
- RT-PCR and other molecular assays are generally more expensive than other influenza tests
- Some molecular assays may not specifically identify all currently circulating influenza A virus subtypes. Depending on the test, a negative result for one influenza A virus subtype may not preclude infection with another influenza A virus subtype.
- Some influenza molecular assays being used are not FDA-cleared and an evaluation has not been performed to assess the accuracy of all available RT-PCR and molecular assays. A list of FDA-cleared tests is available in Table 1. FDA-cleared RT-PCR Assays and Other Molecular Assays for Influenza Viruses [202 KB, 3 pages] .

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Rapid Molecular Assays

Rapid molecular assays are a new type of molecular influenza diagnostic test. These platforms use isothermal nucleic acid amplification and have high sensitivity and yield results in 15 minutes. Currently, there is only one rapid molecular assay that FDA-cleared in the United States. Additional rapid molecular assays may become available in the future. As with other molecular diagnostic tests, if treatment is clinically indicated, antiviral treatment should NOT be withheld from patients with suspected influenza while awaiting testing results during periods of peak influenza activity in the community when the likelihood of influenza is high. More information about antiviral treatment of influenza is available at Antiviral Drugs, Information for Health Care Professionals.

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Additional Information

- Antiviral Agents for the Treatment and Chemoprophylaxis of Influenza [1 MB, 28 pages] (http://www.cdc.gov/mmwr/pdf/rr/rr6001.pdf). Recommendations of the Advisory Committee on Immunization Practices (ACIP).
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- World Health Organization. Clinical management of human infection with pandemic (H1N1) 2009: revised guidance [304 KB, 15 pages] (http://www.who.int/csr/resources/publications/swineflu/clinical_management_h1n1.pdf).
- WHO Global Influenza Surveillance Network. Manual for the laboratory diagnosis and virological surveillance of influenza [2 MB, 153 pages] (http://whqlibdoc.who.int/publications/2011/9789241548090_eng.pdf).

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Table 1. FDA-cleared RT-PCR Assays and Other Molecular Assays for Influenza Viruses

Products	Manufacturer (s)	Influenza Virus Type Detected	Influenza Virus Subtype (s) Differentiated	Other Respiratory Viruses Differentiated	Acceptable Specimens1	Test Time2/ Complexity3
Alere i NAT Flu A/B	Alere	Influenza A and B	None	None	Nasal swabs (Direct)	0.25 h/ CLIA-Waived

CDC Human Influenza Virus Real-Time RT-PCR Diagnostic Panel Influenza A/B Typing Kit4	CDC Influenza Division	Influenza A and B	None	None	Nasopharyngeal swabs, nasal swabs, nasal aspirates, nasal washes, dual nasopharyngeal/ throat swabs, broncheoalveolar lavages, tracheal aspirates, bronchial washes, and viral culture	~4 h/ High
CDC Human Influenza Virus Real-Time RT-PCR Diagnostic Panel Influenza A Subtyping Kit4	CDC Influenza Division	Influenza A	A/H1, A/H3, A/2009 H1	None	Nasopharyngeal swabs, nasal swabs, nasal aspirates, nasal washes, dual nasopharyngeal/ throat swabs, broncheoalveolar lavages, tracheal aspirates, bronchial washes, and viral culture	~4 h/ High
CDC Human Influenza Virus Real-Time RT-PCR Diagnostic Panel Influenza A/H5 (Asian lineage) Kit4	CDC Influenza Division	Influenza A	A/H5N1 (Asian lineage)	None	Nasopharyngeal swabs, nasal swabs, nasal aspirates, nasal washes, dual nasopharyngeal/ throat swabs, broncheoalveolar lavages, tracheal aspirates, bronchial washes, and viral culture	~4 h/ High

CDC Human Influenza Virus Real-Time RT-PCR Diagnostic Panel Influenza B Lineage Genotyping Assay4	CDC Influenza Division	Influenza B	B/Victoria and B/Yamagata lineages	None	Nasopharyngeal swabs, nasal swabs, nasal aspirates, nasal washes, dual nasopharyngeal/ throat swabs	~4 h/ High
Cepheid Xpert Flu Assay	Cepheid	Influenza A and B	A/2009 H1	None	Nasopharyngeal swabs, nasal aspirates, and nasal washes.	1.0 h/ Moderate
Cepheid Xpert Flu/RSV XC Assay	Cepheid	Influenza A and B	None	Respiratory Syncytial Virus	Nasopharyngeal swabs and nasal wash and nasal aspirate (in VTM)	<1.0 h/ Moderate
eSensor® Respiratory Viral Panel (RVP)	Clinical Micro Sensors, Inc. dba GenMark Diagnostics, Inc.	Influenza A and B	A/HI, A/H3, A/2009 H1	Respiratory Syncytial Virus subtype A, Respiratory Syncytial Virus subtype B, Parainfluenza 1, 2, and 3 virus, Human Metapneumovirus, Adenovirus Species B/E, Adenovirus Species C, and Human Rhinovirus	Nasopharyngeal swabs	~8 h/ High
FilmArray Respiratory Panel	Idaho Technologies	Influenza A and B	A/HI, A/H3, A/2009 H1	Respiratory Syncytial Virus, Parainfluenza 1, 2, 3 and 4 virus, Human Metapneumovirus, Rhinovirus/Enterovirus, Adenovirus, Coronavirus HKU1, Coronavirus NL63	Nasopharyngeal swabs	1.0 h/ Moderate

Ibis PLEX-ID Flu	Ibis/Abbott	Influenza A and B	A/HI, A/H3, A/2009 H1	None	Nasopharyngeal swabs	~8 h/ High
IMDx Flu A/B and RSV for Abbott *m*2000	IMDx	Influenza A and B	A/HI, A/H3, A/2009 H1	None	Nasopharyngeal swabs	~8 h/ High
IQuum Liat Influenza A/B Assay	IQuum	Influenza A and B	None	None	Nasopharyngeal swabs	0.5 h/ Moderate
Prodesse PROFLU™+	GenProbe	Influenza A and B	None	Respiratory Syncytial Virus	Nasopharyngeal swabs	<4h/ High
Prodesse ProFAST™+	GenProbe	Influenza A	A/HI, A/H3, A/2009 H1	None	Nasopharyngeal swabs	<4h/ High
Quidel Molecular Influenza A+B Assay	Quidel	Influenza A and B	None	None	Nasopharyngeal swabs and nasal swabs	~4 h/ High
Qiagen Artus Influenza A/B Rotor-gene RT-PCR kit	Qiagen	Influenza A and B	None	None	Nasopharyngeal swabs	~4 h/ High
Simplexa™ Flu A/B & RSV	Focus Diagnostics, 3M	Influenza A and B	None	RSV	Nasopharyngeal swabs	<4h/ High
Simplexa™ Flu A/B & RSV Direct	Focus Diagnostics, 3M	Influenza A and B	None	RSV	Nasopharyngeal swabs	<2h/ Moderate
Simplexa™ Influenza A H1N1 (2009)	Focus Diagnostics, 3M	Influenza A	A/2009 H1	None	Nasopharyngeal swabs, nasal swabs, and nasopharyngeal aspirates	<4h/ High

U.S. Army JBAIDS Influenza A&B Detection Kit4	Idaho Technologies	Influenza A and B	None	None	Nasopharyngeal swabs and Nasopharyngeal washes	~4 h/ High
U.S. Army JBAIDS Influenza A Subtyping Kit4	Idaho Technologies	Influenza A	A/HI, A/H3, A/2009 H1	None	Nasopharyngeal swabs and Nasopharyngeal washes	~4 h/ High
U.S. Army JBAIDS Influenza A/H5 Kit4	Idaho Technologies	Influenza A	A/H5N1 (Asian Lineage)	None	Nasopharyngeal and throat swabs	~4 h/ High
Verigene® Respiratory Virus Nucleic Acid Test	Nanosphere, Inc	Influenza A and B	None	Respiratory Syncytial Virus subtype A, Respiratory Syncytial Virus subtype B	Nasopharyngeal swabs	3.5 h/ Moderate
Verigene® Respiratory Virus Plus Nucleic Acid Test (RV+)	Nanosphere, Inc	Influenza A and B	A/HI, A/H3, A/2009 H1	Respiratory Syncytial Virus subtype A, Respiratory Syncytial Virus subtype B	Nasopharyngeal swabs	3.5 h/ Moderate
x-TAG® Respiratory Viral Panel (RVP)	Luminex Molecular Diagnostics Inc.	Influenza A and B	A/H1, A/H3	Respiratory Syncytial Virus subtype A, Respiratory Syncytial Virus subtype B, Parainfluenza 1, 2, and 3 virus, Human Metapneumovirus, Rhinovirus, and Adenovirus	Nasopharyngeal swabs	~8 h/ High

x-TAG® Respiratory Viral Panel Fast (RVP FAST))	Luminex Molecular Diagnostics Inc.	Influenza A and B	A/H1, A/H3	Respiratory Syncytial Virus Human Metapneumovirus, Rhinovirus, and Adenovirus	Nasopharyngeal swabs	~6 h/ High

Printer-friendly Version PDF [114 KB, 3 pages]

1. These specimen types are specified in product package inserts cleared by the U.S. Food and Drug Administration (FDA)
2. Test Time is inclusive of actual test time and is exclusive of transport, handling, laboratory run schedules, and generating results. Timing may vary depending on extraction process used. Contact laboratory for expected turn-around time.
3. Clinical Laboratory Improvement Amendments require categorization of tests as waived, moderate or high complexity. Ref: http://www.cms.gov/Regulations-and-Guidance/Legislation/CLIA/index.html (http://www.cms.gov/Regulations-and-Guidance/Legislation/CLIA/index.html)
4. Available only to qualified DoD laboratories, U.S. public health laboratories, and NREVSS collaborating laboratories.

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File Formats Help:
How do I view different file formats (PDF, DOC, PPT, MPEG) on this site? (http://www.cdc.gov/Other/plugins/)

(http://www.cdc.gov/Other/plugins/#pdf)

Page last reviewed: February 18, 2015
Page last updated: February 18, 2015
Content source: Centers for Disease Control and Prevention (http://www.cdc.gov/)

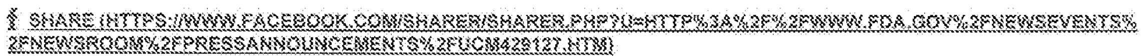
EXHIBIT 2

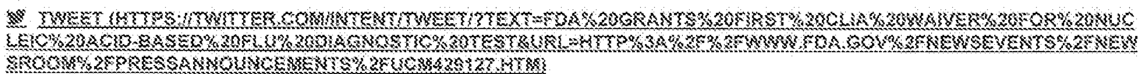
(http://www.fda.gov/NewsEvents/Newsroom/PressAnnouncements/ucm429127.htm)

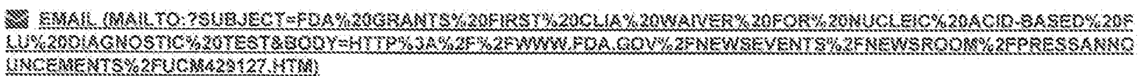
U.S. Food and Drug Administration
Protecting and Promoting *Your* Health

FDA News Release

FDA grants first CLIA waiver for nucleic acid-based flu diagnostic test







For Immediate Release

January 6, 2015

Release

The U.S. Food and Drug Administration today granted the first waiver to allow a nucleic acid-based test, the Alere i Influenza A & B test, to be used in a greater variety of health care settings. The test was previously only available for use in certain laboratories.

Influenza, commonly known as the flu, is a contagious respiratory illness caused by two types of influenza viruses: Type A and Type B. Flu infections can range from mild to severe and can sometimes lead to hospitalization and death. According to the Centers for Disease Control and Prevention, more than 200,000 people in the United States are hospitalized from seasonal flu-related complications each year.

Because the FDA granted a waiver under the Clinical Laboratory Improvement Amendments (CLIA), the Alere i Influenza A & B test can be distributed to a broad variety of non-traditional laboratory sites, including physicians' offices, emergency rooms, health department clinics, and other health care facilities.

"Today's decision allows the first nucleic acid-based test to be available in clinical settings that previously could not use this technology," said Alberto Gutierrez, Ph.D., director of the Office of In Vitro Diagnostics and Radiological Health in the FDA's Center for Devices and Radiological Health. "We expect many other simple and accurate tests using nucleic acid-based technology to be developed in the near future. Once cleared by FDA, such tests can allow health care professionals to receive test results more quickly to inform further diagnostic and treatment decisions."

The Alere i Influenza A & B test uses a nasal swab sample from a patient with signs and symptoms of flu infection. The test provides results in as little as 15 minutes and may be performed in the presence of the patient. Negative results do not rule out influenza virus infection; the test is intended to aid in diagnosis along with the evaluation of other risk factors.

The FDA's waiver is related to CLIA, federal standards that apply to clinical laboratory testing on humans, with certain exceptions. The FDA first cleared the Alere i Influenza A & B test in June 2014 as a prescription-only device to detect influenza A and B viral RNA in nasal swab samples and categorized it under CLIA as moderate complexity. The type of CLIA certificate a laboratory obtains depends upon the complexity of the tests it performs. CLIA regulations describe three levels of test complexity: waived tests, moderate complexity tests and high complexity tests.

The FDA granted a waiver under CLIA for the Alere i Influenza A & B test after the manufacturer submitted data demonstrating the test's ease of use and low risk of false results when used by untrained operators. This is critical if the test is to be allowed for use outside of moderate- and high-complexity laboratories. The agency reviewed clinical study data from more than 500 patients with signs and symptoms of respiratory viral infection tested for influenza using both the Alere i Influenza A & B test and an FDA-cleared molecular comparator. Compared to the FDA-cleared comparator method, the Alere i Influenza A & B test demonstrated high accuracy when identifying patients with or without influenza A and influenza B by users untrained in laboratory procedures.

The Alere i Influenza A & B test is manufactured by Alere Scarborough, Inc., located in Scarborough, Maine.

The FDA, an agency within the U.S. Department of Health and Human Services, protects the public health by assuring the safety, effectiveness, and security of human and veterinary drugs, vaccines and other biological products for human use, and medical devices. The agency also is responsible for the safety and security of our nation's food supply, cosmetics, dietary supplements, products that give off electronic radiation, and for regulating tobacco products.

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Inquiries

Media

Jennifer Haliski (mailto:jennifer.haliski@fda.hhs.gov)
301-796-0776

Consumers

888-INFO-FDA

Related Information

- FDA: Medical Devices (/MedicalDevices/default.htm)
- Office of In Vitro Diagnostics and Radiological Health (/AboutFDA/CentersOffices/OfficeofMedicalProductsandTobacco/CDRH/CDRHOffices/ucm115904.htm)
- FDA: CLIA – Test waived by FDA from January 2000 to present (http://www.accessdata.fda.gov/scripts/cdrh/cfdocs/cfClia/testswaived.cfm)
- CDC: Influenza (Flu) (http://www.cdc.gov/flu/index.htm)

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More in Press Announcements (/NewsEvents/Newsroom/PressAnnouncements/default.htm)

2014 (/NewsEvents/Newsroom/PressAnnouncements/2014/default.htm)

2013 (/NewsEvents/Newsroom/PressAnnouncements/2013/default.htm)

Electronic Patent Application Fee Transmittal

Application Number:	14067620
Filing Date:	30-Oct-2013
Title of Invention:	Nicking and Extension Amplification Reaction for the Exponential Amplification of Nucleic Acids
First Named Inventor/Applicant Name:	Brian K. Maples
Filer:	Ian J.S. Lodovice/Mary Florczak
Attorney Docket Number:	30171-0025002 / ITI-001

Filed as Large Entity

Filing Fees for **Utility under 35 USC 111(a)**

Description	Fee Code	Quantity	Amount	Sub-Total in USD($)
Basic Filing:				
Pages:				
Claims:				
Miscellaneous-Filing:				
Petition:				
Patent-Appeals-and-Interference:				
Post-Allowance-and-Post-Issuance:				
Extension-of-Time:				

Description	Fee Code	Quantity	Amount	Sub-Total in USD($)
Extension - 2 months with $0 paid	1252	1	600	600
Miscellaneous:				
Request for Continued Examination	1801	1	1200	1200
	Total in USD ($)			**1800**

Electronic Acknowledgement Receipt

EFS ID:	23037894
Application Number:	14067620
International Application Number:	
Confirmation Number:	4288
Title of Invention:	Nicking and Extension Amplification Reaction for the Exponential Amplification of Nucleic Acids
First Named Inventor/Applicant Name:	Brian K. Maples
Customer Number:	26161
Filer:	Ian J.S. Lodovice/Mary Florczak
Filer Authorized By:	Ian J.S. Lodovice
Attorney Docket Number:	30171-0025002 / ITI-001
Receipt Date:	27-JUL-2015
Filing Date:	30-OCT-2013
Time Stamp:	20:35:15
Application Type:	Utility under 35 USC 111(a)

Payment information:

Submitted with Payment	yes
Payment Type	Deposit Account
Payment was successfully received in RAM	$1800
RAM confirmation Number	18765
Deposit Account	061050
Authorized User	FISH & RICHARDSON P C

The Director of the USPTO is hereby authorized to charge indicated fees and credit any overpayment as follows:

File Listing:

Document Number	Document Description	File Name	File Size(Bytes)/ Message Digest	Multi Part /.zip	Pages (if appl.)
1	Request for Continued Examination (RCE)	30171RCE.pdf	141632 90a9f3682de4cd23a072943b2a139615fdb2a4e0	no	1

Warnings:

This is not a USPTO supplied RCE SB30 form.

Information:

Document Number	Document Description	File Name	File Size(Bytes)/ Message Digest	Multi Part /.zip	Pages (if appl.)
2		30171Response.pdf	2759776 dc6d52c1fe4664ad0ddb17a44849729b88f10ff7	yes	30

Multipart Description/PDF files in .zip description

Document Description	Start	End
Response After Final Action	1	1
Claims	2	8
Applicant Arguments/Remarks Made in an Amendment	9	30

Warnings:

Information:

Document Number	Document Description	File Name	File Size(Bytes)/ Message Digest	Multi Part /.zip	Pages (if appl.)
3	Fee Worksheet (SB06)	fee-info.pdf	32475 db71148503cc93c0157a83e35312c9fdfe1b9126	no	2

Warnings:

Information:

Total Files Size (in bytes):	2933883

This Acknowledgement Receipt evidences receipt on the noted date by the USPTO of the indicated documents, characterized by the applicant, and including page counts, where applicable. It serves as evidence of receipt similar to a Post Card, as described in MPEP 503.

New Applications Under 35 U.S.C. 111
If a new application is being filed and the application includes the necessary components for a filing date (see 37 CFR 1.53(b)-(d) and MPEP 506), a Filing Receipt (37 CFR 1.54) will be issued in due course and the date shown on this Acknowledgement Receipt will establish the filing date of the application.

National Stage of an International Application under 35 U.S.C. 371
If a timely submission to enter the national stage of an international application is compliant with the conditions of 35 U.S.C. 371 and other applicable requirements a Form PCT/DO/EO/903 indicating acceptance of the application as a national stage submission under 35 U.S.C. 371 will be issued in addition to the Filing Receipt, in due course.

New International Application Filed with the USPTO as a Receiving Office
If a new international application is being filed and the international application includes the necessary components for an international filing date (see PCT Article 11 and MPEP 1810), a Notification of the International Application Number and of the International Filing Date (Form PCT/RO/105) will be issued in due course, subject to prescriptions concerning national security, and the date shown on this Acknowledgement Receipt will establish the international filing date of the application.

PTO/SB/06 (09-11)
Approved for use through 1/31/2014. OMB 0651-0032
U.S. Patent and Trademark Office; U.S. DEPARTMENT OF COMMERCE
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PATENT APPLICATION FEE DETERMINATION RECORD Substitute for Form PTO-875	Application or Docket Number 14/067,620	Filing Date 10/30/2013	☐ To be Mailed

ENTITY: ☒ LARGE ☐ SMALL ☐ MICRO

APPLICATION AS FILED – PART I

FOR	(Column 1) NUMBER FILED	(Column 2) NUMBER EXTRA	RATE ($)	FEE ($)
☐ BASIC FEE (37 CFR 1.16(a), (b), or (c))	N/A	N/A	N/A	
☐ SEARCH FEE (37 CFR 1.16(k), (i), or (m))	N/A	N/A	N/A	
☐ EXAMINATION FEE (37 CFR 1.16(o), (p), or (q))	N/A	N/A	N/A	
TOTAL CLAIMS (37 CFR 1.16(i))	minus 20 =	*	X $ =	
INDEPENDENT CLAIMS (37 CFR 1.16(h))	minus 3 =	*	X $ =	
☐ APPLICATION SIZE FEE (37 CFR 1.16(s))	If the specification and drawings exceed 100 sheets of paper, the application size fee due is $310 ($155 for small entity) for each additional 50 sheets or fraction thereof. See 35 U.S.C. 41(a)(1)(G) and 37 CFR 1.16(s).			
☐ MULTIPLE DEPENDENT CLAIM PRESENT (37 CFR 1.16(j))				
* If the difference in column 1 is less than zero, enter "0" in column 2.			TOTAL	

APPLICATION AS AMENDED – PART II

AMENDMENT 07/27/2015	(Column 1) CLAIMS REMAINING AFTER AMENDMENT		(Column 2) HIGHEST NUMBER PREVIOUSLY PAID FOR	(Column 3) PRESENT EXTRA	RATE ($)	ADDITIONAL FEE ($)
Total (37 CFR 1.16(i))	* 36	Minus	** 36	= 0	x $80 =	0
Independent (37 CFR 1.16(h))	* 3	Minus	***3	= 0	x $420 =	0
☐ Application Size Fee (37 CFR 1.16(s))						
☐ FIRST PRESENTATION OF MULTIPLE DEPENDENT CLAIM (37 CFR 1.16(j))						
					TOTAL ADD'L FEE	**0**

AMENDMENT	(Column 1) CLAIMS REMAINING AFTER AMENDMENT		(Column 2) HIGHEST NUMBER PREVIOUSLY PAID FOR	(Column 3) PRESENT EXTRA	RATE ($)	ADDITIONAL FEE ($)
Total (37 CFR 1.16(i))	*	Minus	**	=	X $ =	
Independent (37 CFR 1.16(h))	*	Minus	***	=	X $ =	
☐ Application Size Fee (37 CFR 1.16(s))						
☐ FIRST PRESENTATION OF MULTIPLE DEPENDENT CLAIM (37 CFR 1.16(j))						
					TOTAL ADD'L FEE	

* If the entry in column 1 is less than the entry in column 2, write "0" in column 3.
** If the "Highest Number Previously Paid For" IN THIS SPACE is less than 20, enter "20".
*** If the "Highest Number Previously Paid For" IN THIS SPACE is less than 3, enter "3".
The "Highest Number Previously Paid For" (Total or Independent) is the highest number found in the appropriate box in column 1.

LIE
/MARISSA BLYTHER/

This collection of information is required by 37 CFR 1.16. The information is required to obtain or retain a benefit by the public which is to file (and by the USPTO to process) an application. Confidentiality is governed by 35 U.S.C. 122 and 37 CFR 1.14. This collection is estimated to take 12 minutes to complete, including gathering, preparing, and submitting the completed application form to the USPTO. Time will vary depending upon the individual case. Any comments on the amount of time you require to complete this form and/or suggestions for reducing this burden, should be sent to the Chief Information Officer, U.S. Patent and Trademark Office, U.S. Department of Commerce, P.O. Box 1450, Alexandria, VA 22313-1450. DO NOT SEND FEES OR COMPLETED FORMS TO THIS ADDRESS. **SEND TO: Commissioner for Patents, P.O. Box 1450, Alexandria, VA 22313-1450.**

If you need assistance in completing the form, call 1-800-PTO-9199 and select option 2.

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APPLICATION NO.	FILING DATE	FIRST NAMED INVENTOR	ATTORNEY DOCKET NO.	CONFIRMATION NO.
14/067,620	10/30/2013	Brian K. Maples	30171-0025002 / ITI-001	4288

26161 7590 06/17/2015
FISH & RICHARDSON P.C. (BO)
P.O. BOX 1022
MINNEAPOLIS, MN 55440-1022

EXAMINER	
BERTAGNA, ANGELA MARIE	

ART UNIT	PAPER NUMBER
1637	

NOTIFICATION DATE	DELIVERY MODE
06/17/2015	ELECTRONIC

Please find below and/or attached an Office communication concerning this application or proceeding.

The time period for reply, if any, is set in the attached communication.

Notice of the Office communication was sent electronically on above-indicated "Notification Date" to the following e-mail address(es):

PATDOCTC@fr.com

PTOL-90A (Rev. 04/07)

Applicant-Initiated Interview Summary	**Application No.** 14/067,620	**Applicant(s)** MAPLES ET AL.	
	Examiner Angela M. Bertagna	**Art Unit** 1637	

All participants (applicant, applicant's representative, PTO personnel):

(1) *Angela M. Bertagna*.

(2) *Ian Lodovice (Applicant's representative)*.

(3) *Belinda Lew (Applicant's representative)*.

(4) *Richard Roth (Applicant)*.

Date of Interview: *12 June 2015*.

Type: ☒ Telephonic ☐ Video Conference
☐ Personal [copy given to: ☐ applicant ☐ applicant's representative]

Exhibit shown or demonstration conducted: ☐ Yes ☒ No.
If Yes, brief description: _____.

Issues Discussed ☐101 ☒112 ☐102 ☒103 ☐Others
(For each of the checked box(es) above, please describe below the issue and detailed description of the discussion)

Claim(s) discussed: *67-73,76,77,80,83,85-88,90-92 and 96-106*.

Identification of prior art discussed: *Wick et al. (US 6,063,604), Kong et al. (US 6,191,267), and Yao et al. (US 2009/0092967 A1), all of which are of record and were cited in the last office action*.

Substance of Interview
(For each issue discussed, provide a detailed description and indicate if agreement was reached. Some topics may include: identification or clarification of a reference or a portion thereof, claim interpretation, proposed amendments, arguments of any applied references etc...)

We discussed the new matter rejection of claims 67-73, 76, 77, 80, 83, and 85-92 made in the final rejection, and specifically, whether the specification provides support for omission of an amplification-associated denaturation step of any type. We also discussed claim language that could overcome the new matter rejection. Agreement was not reached concerning this issue. We also discussed the proposed amendment concerning the amplication times and yields. The examiner stated that the cited references do not appear to suggest amplification times of 1-20 minutes, but that further search and consideration would be required. The examiner also suggested incorporating a discussion of why the claimed amplification times are not obvious (i.e., not merely routine optimization of a results-effective variable) into the next response.

Applicant recordation instructions: The formal written reply to the last Office action must include the substance of the interview. (See MPEP section 713.04). If a reply to the last Office action has already been filed, applicant is given a non-extendable period of the longer of one month or thirty days from this interview date, or the mailing date of this interview summary form, whichever is later, to file a statement of the substance of the interview

Examiner recordation instructions: Examiners must summarize the substance of any interview of record. A complete and proper recordation of the substance of an interview should include the items listed in MPEP 713.04 for complete and proper recordation including the identification of the general thrust of each argument or issue discussed, a general indication of any other pertinent matters discussed regarding patentability and the general results or outcome of the interview, to include an indication as to whether or not agreement was reached on the issues raised.

☐ Attachment

/Angela M. Bertagna/
Primary Examiner, Art Unit 1637

Summary of Record of Interview Requirements

Manual of Patent Examining Procedure (MPEP), Section 713.04, Substance of Interview Must be Made of Record
A complete written statement as to the substance of any face-to-face, video conference, or telephone interview with regard to an application must be made of record in the application whether or not an agreement with the examiner was reached at the interview.

Title 37 Code of Federal Regulations (CFR) § 1.133 Interviews
Paragraph (b)

In every instance where reconsideration is requested in view of an interview with an examiner, a complete written statement of the reasons presented at the interview as warranting favorable action must be filed by the applicant. An interview does not remove the necessity for reply to Office action as specified in §§ 1.111, 1.135. (35 U.S.C. 132)

37 CFR §1.2 Business to be transacted in writing.
All business with the Patent or Trademark Office should be transacted in writing. The personal attendance of applicants or their attorneys or agents at the Patent and Trademark Office is unnecessary. The action of the Patent and Trademark Office will be based exclusively on the written record in the Office. No attention will be paid to any alleged oral promise, stipulation, or understanding in relation to which there is disagreement or doubt.

The action of the Patent and Trademark Office cannot be based exclusively on the written record in the Office if that record is itself incomplete through the failure to record the substance of interviews.

It is the responsibility of the applicant or the attorney or agent to make the substance of an interview of record in the application file, unless the examiner indicates he or she will do so. It is the examiner's responsibility to see that such a record is made and to correct material inaccuracies which bear directly on the question of patentability.

Examiners must complete an Interview Summary Form for each interview held where a matter of substance has been discussed during the interview by checking the appropriate boxes and filling in the blanks. Discussions regarding only procedural matters, directed solely to restriction requirements for which interview recordation is otherwise provided for in Section 812.01 of the Manual of Patent Examining Procedure, or pointing out typographical errors or unreadable script in Office actions or the like, are excluded from the interview recordation procedures below. Where the substance of an interview is completely recorded in an Examiners Amendment, no separate Interview Summary Record is required.

The Interview Summary Form shall be given an appropriate Paper No., placed in the right hand portion of the file, and listed on the "Contents" section of the file wrapper. In a personal interview, a duplicate of the Form is given to the applicant (or attorney or agent) at the conclusion of the interview. In the case of a telephone or video-conference interview, the copy is mailed to the applicant's correspondence address either with or prior to the next official communication. If additional correspondence from the examiner is not likely before an allowance or if other circumstances dictate, the Form should be mailed promptly after the interview rather than with the next official communication.

The Form provides for recordation of the following information:
– Application Number (Series Code and Serial Number)
– Name of applicant
– Name of examiner
– Date of interview
– Type of interview (telephonic, video-conference, or personal)
– Name of participant(s) (applicant, attorney or agent, examiner, other PTO personnel, etc.)
– An indication whether or not an exhibit was shown or a demonstration conducted
– An identification of the specific prior art discussed
– An indication whether an agreement was reached and if so, a description of the general nature of the agreement (may be by attachment of a copy of amendments or claims agreed as being allowable). Note: Agreement as to allowability is tentative and does not restrict further action by the examiner to the contrary.
– The signature of the examiner who conducted the interview (if Form is not an attachment to a signed Office action)

It is desirable that the examiner orally remind the applicant of his or her obligation to record the substance of the interview of each case. It should be noted, however, that the Interview Summary Form will not normally be considered a complete and proper recordation of the interview unless it includes, or is supplemented by the applicant or the examiner to include, all of the applicable items required below concerning the substance of the interview.

A complete and proper recordation of the substance of any interview should include at least the following applicable items:
1) A brief description of the nature of any exhibit shown or any demonstration conducted,
2) an identification of the claims discussed,
3) an identification of the specific prior art discussed,
4) an identification of the principal proposed amendments of a substantive nature discussed, unless these are already described on the Interview Summary Form completed by the Examiner,
5) a brief identification of the general thrust of the principal arguments presented to the examiner,
   (The identification of arguments need not be lengthy or elaborate. A verbatim or highly detailed description of the arguments is not required. The identification of the arguments is sufficient if the general nature or thrust of the principal arguments made to the examiner can be understood in the context of the application file. Of course, the applicant may desire to emphasize and fully describe those arguments which he or she feels were or might be persuasive to the examiner.)
6) a general indication of any other pertinent matters discussed, and
7) if appropriate, the general results or outcome of the interview unless already described in the Interview Summary Form completed by the examiner.

Examiners are expected to carefully review the applicant's record of the substance of an interview. If the record is not complete and accurate, the examiner will give the applicant an extendable one month time period to correct the record.

Examiner to Check for Accuracy

If the claims are allowable for other reasons of record, the examiner should send a letter setting forth the examiner's version of the statement attributed to him or her. If the record is complete and accurate, the examiner should place the indication, "Interview Record OK" on the paper recording the substance of the interview along with the date and the examiner's initials.

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APPLICATION NO.	FILING DATE	FIRST NAMED INVENTOR	ATTORNEY DOCKET NO.	CONFIRMATION NO.
14/067,620	10/30/2013	Brian K. Maples	30171-0025002 / ITI-001	4288

26161 7590 06/11/2015
FISH & RICHARDSON P.C. (BO)
P.O. BOX 1022
MINNEAPOLIS, MN 55440-1022

EXAMINER	
BERTAGNA, ANGELA MARIE	

ART UNIT	PAPER NUMBER
1637	

NOTIFICATION DATE	DELIVERY MODE
06/11/2015	ELECTRONIC

Please find below and/or attached an Office communication concerning this application or proceeding.

The time period for reply, if any, is set in the attached communication.

Notice of the Office communication was sent electronically on above-indicated "Notification Date" to the following e-mail address(es):

PATDOCTC@fr.com

PTOL-90A (Rev. 04/07)

Advisory Action Before the Filing of an Appeal Brief	Application No. 14/067,620	Applicant(s) MAPLES ET AL.	
	Examiner Angela M. Bertagna	Art Unit 1637	AIA (First Inventor to File) Status No

--The MAILING DATE of this communication appears on the cover sheet with the correspondence address --

THE REPLY FILED 27 May 2015 FAILS TO PLACE THIS APPLICATION IN CONDITION FOR ALLOWANCE.

NO NOTICE OF APPEAL FILED

1. ☒ The reply was filed after a final rejection. No Notice of Appeal has been filed. To avoid abandonment of this application, applicant must timely file one of the following replies: (1) an amendment, affidavit, or other evidence, which places the application in condition for allowance; (2) a Notice of Appeal (with appeal fee) in compliance with 37 CFR 41.31; or (3) a Request for Continued Examination (RCE) in compliance with 37 CFR 1.114 if this is a utility or plant application. Note that RCEs are not permitted in design applications. The reply must be filed within one of the following time periods:
   a) ☒ The period for reply expires 3 months from the mailing date of the final rejection.
   b) ☐ The period for reply expires on: (1) the mailing date of this Advisory Action; or (2) the date set forth in the final rejection, whichever is later. In no event, however, will the statutory period for reply expire later than SIX MONTHS from the mailing date of the final rejection.
   c) ☐ A prior Advisory Action was mailed more than 3 months after the mailing date of the final rejection in response to a first after-final reply filed within 2 months of the mailing date of the final rejection. The current period for reply expires months from the mailing date of *the prior Advisory Action* or SIX MONTHS from the mailing date of the final rejection, whichever is earlier.
   Examiner Note: If box 1 is checked, check either box (a), (b) or (c). ONLY CHECK BOX (b) WHEN THIS ADVISORY ACTION IS THE FIRST RESPONSE TO APPLICANT'S FIRST AFTER-FINAL REPLY WHICH WAS FILED WITHIN TWO MONTHS OF THE FINAL REJECTION. ONLY CHECK BOX (c) IN THE LIMITED SITUATION SET FORTH UNDER BOX (c). See MPEP 706.07(f).

Extensions of time may be obtained under 37 CFR 1.136(a). The date on which the petition under 37 CFR 1.136(a) and the appropriate extension fee have been filed is the date for purposes of determining the period of extension and the corresponding amount of the fee. The appropriate extension fee under 37 CFR 1.17(a) is calculated from: (1) the expiration date of the shortened statutory period for reply originally set in the final Office action; or (2) as set forth in (b) or (c) above, if checked. Any reply received by the Office later than three months after the mailing date of the final rejection, even if timely filed, may reduce any earned patent term adjustment. See 37 CFR 1.704(b).

NOTICE OF APPEAL

2. ☐ The Notice of Appeal was filed on _____. A brief in compliance with 37 CFR 41.37 must be filed within two months of the date of filing the Notice of Appeal (37 CFR 41.37(a)), or any extension thereof (37 CFR 41.37(e)), to avoid dismissal of the appeal. Since a Notice of Appeal has been filed, any reply must be filed within the time period set forth in 37 CFR 41.37(a).

AMENDMENTS

3. ☒ The proposed amendments filed after a final rejection, but prior to the date of filing a brief, will not be entered because
   a) ☒ They raise new issues that would require further consideration and/or search (see NOTE below);
   b) ☐ They raise the issue of new matter (see NOTE below);
   c) ☒ They are not deemed to place the application in better form for appeal by materially reducing or simplifying the issues for appeal; and/or
   d) ☒ They present additional claims without canceling a corresponding number of finally rejected claims.
   NOTE: *See Continuation Sheet*. (See 37 CFR 1.116 and 41.33(a)).
4. ☐ The amendments are not in compliance with 37 CFR 1.121. See attached Notice of Non-Compliant Amendment (PTOL-324).
5. ☐ Applicant's reply has overcome the following rejection(s): _____.
6. ☐ Newly proposed or amended claim(s) _____ would be allowable if submitted in a separate, timely filed amendment canceling the non-allowable claim(s).
7. ☒ For purposes of appeal, the proposed amendment(s): (a) ☒ will not be entered, or (b) ☐ will be entered, and an explanation of how the new or amended claims would be rejected is provided below or appended.

AFFIDAVIT OR OTHER EVIDENCE

8. ☐ A declaration(s)/affidavit(s) under **37 CFR 1.130(b)** was/were filed on _____.
9. ☐ The affidavit or other evidence filed after final action, but before or on the date of filing a Notice of Appeal will not be entered because applicant failed to provide a showing of good and sufficient reasons why the affidavit or other evidence is necessary and was not earlier presented. See 37 CFR 1.116(e).
10. ☐ The affidavit or other evidence filed after the date of filing the Notice of Appeal, but prior to the date of filing a brief, will not be entered because the affidavit or other evidence failed to overcome all rejections under appeal and/or appellant fails to provide a showing of good and sufficient reasons why it is necessary and was not earlier presented. See 37 CFR 41.33(d)(1).
11. ☐ The affidavit or other evidence is entered. An explanation of the status of the claims after entry is below or attached.

REQUEST FOR RECONSIDERATION/OTHER

12. ☒ The request for reconsideration has been considered but does NOT place the application in condition for allowance because: See Continuation Sheet.
13. ☐ Note the attached Information *Disclosure Statement*(s). (PTO/SB/08) Paper No(s). _____
14. ☒ Other: PTO-2323 attached.

STATUS OF CLAIMS

15. The status of the claim(s) is (or will be) as follows:
   Claim(s) allowed: .
   Claim(s) objected to: .
   Claim(s) rejected: 67-73,76,77,80,83 and 85-92.
   Claim(s) withdrawn from consideration: 74,75,78,79,81,82 and 95.

/Angela M. Bertagna/
Primary Examiner, Art Unit 1637

Continuation of 3. The proposed amendments require further consideration and search because the requirement for a narrower amplification time range (claim 67 and new claims 99-106) has not been presented previously. Also, the amplification yields recited in new claims 96-98 have not been presented previously and require further consideration and search.

Continuation of 12. Applicant's arguments filed on 5/27/15 have been fully considered to the extent that they are not directed to the proposed amendment.

Applicant's arguments on pages 9-10 regarding the new matter rejection of claims 67-73, 76 ,77, 80, 83, and 85-92 have been fully considered, but they were not persuasive. In contrast to Applicant's arguments, the ordinary artisan would not have considered the original disclosure to provide clear support for omitting any denaturation step. There is no discussion of omitting any type of denaturation step other than a heat denaturation step. There is also no clear indication in the specification that omitting any other type of denaturation step was contemplated. In the absence of such disclosure, it must be concluded that the applicant was not in possession of the genus encompassed by the claims. The rejection has been maintained.

Applicant's arguments on pages 11-12 regarding the rejection of claims 67-73, 76, 77, 80, 83, and 85-92 under pre-AIA 35 U.S.C. 103(a) citing Wick, Kong, and Yao are directed to the proposed amendment. Since the amendment was not entered, the arguments are moot. The claims remain rejected for the reasons set forth in the final rejection.

Regarding the provisional obviousness-type double patenting rejections citing co-pending Application Serial Nos. 14/067,623, 11/778,018, and 12/173,020, Applicant requests that the issue be held in abeyance until the indication of allowable subject matter (pages 12-13). Applicant's request is noted. The rejections are maintained since the proposed amendment was not entered and the rejections made in the final rejection are not limited to provisional obviousness-type double patenting rejections.

AFCP 2.0 Decision	**Application No.** 14/067,620	**Applicant(s)** MAPLES ET AL.
	Examiner Angela M. Bertagna	**Art Unit** 1637

This is in response to the After Final Consideration Pilot request filed 27 May 2015.

1. **Improper Request** – The AFCP 2.0 request is improper for the following reason(s) and the after final amendment submitted with the request will be treated under pre-pilot procedure.

   ☐ An AFCP 2.0 request form PTO/SB/434 (or equivalent document) was not submitted.

   ☐ A non-broadening amendment to at least one independent claim was not submitted.

   ☐ A proper AFCP 2.0 request was submitted in response to the most recent final rejection.

   ☐ Other:

2. **Proper Request**

   A. After final amendment submitted with the request will not be treated under AFCP 2.0.
   The after final amendment cannot be reviewed and a search conducted within the guidelines of the pilot program.

   ☒ The after final amendment will be treated under pre-pilot procedure.

   B. Updated search and/or completed additional consideration.
   The examiner performed an updated search and/or completed additional consideration of the after final amendment within the time authorized for the pilot program. The result(s) of the updated search and/or completed additional consideration are:

   ☐ 1. All of the rejections in the most recent final Office action are overcome and a Notice of Allowance is issued herewith.

   ☐ 2. The after final amendment would not overcome all of the rejections in the most recent final Office action. See attached interview summary for further details.

   ☐ 3. The after final amendment was reviewed, and it raises a new issue(s). See attached interview summary for further details.

   ☐ 4. The after final amendment raises new issues, but would overcome all of the rejections in the most recent final Office action. A decision on determining allowability could not be made within the guidelines of the pilot. See attached interview summary for further details, including any newly discovered prior art.

   ☐ 5. Other:

Examiner Note: Please attach an interview summary when necessary as described above.

DO NOT ENTER: /A.B./

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

First Named Inventor : Brian K. Maples
Serial No. : 14/067,620
Filed : October 30, 2013
Art Unit : 1637
Examiner : Angela Marie Bertagna
Conf. No. : 4288
Title : NICKING AND EXTENSION AMPLIFICATION REACTION FOR THE EXPONENTIAL AMPLIFICATION OF NUCLEIC ACIDS

MAIL STOP AF
Commissioner for Patents
P.O. Box 1450
Alexandria, VA 22313-1450

AMENDMENT IN REPLY TO FINAL ACTION OF FEBRUARY 27, 2015

The present amendment is accompanied by a Certification and Request for Consideration Under the After Final Consideration Pilot Program 2.0 (AFCP 2.0).

Please amend the above-identified application as follows.

Doc Code: A.NE.AFCP
Document Description: After Final Consideration Pilot Program Request

PTO/SB/434 (05-13)

CERTIFICATION AND REQUEST FOR CONSIDERATION UNDER THE AFTER FINAL CONSIDERATION PILOT PROGRAM 2.0

Practitioner Docket No.: 30171-0025002	Application No.: 14/067,620	Filing Date: October 30, 2013
First Named Inventor: Brian K. Maples	Title: Nicking and Extension Amplification Reaction for the Exponential Amplification of Nucleic Acids	

APPLICANT HEREBY CERTIFIES THE FOLLOWING AND REQUESTS CONSIDERATION UNDER THE AFTER FINAL CONSIDERATION PILOT PROGRAM 2.0 (AFCP 2.0) OF THE ACCOMPANYING RESPONSE UNDER 37 CFR 1.116.

1. The above-identified application is (i) an original utility, plant, or design nonprovisional application filed under 35 U.S.C. 111(a) [a continuing application (*e.g.*, a continuation or divisional application) is filed under 35 U.S.C. 111(a) and is eligible under (i)], or (ii) an international application that has entered the national stage in compliance with 35 U.S.C. 371(c).
2. The above-identified application contains an outstanding final rejection.
3. Submitted herewith is a response under 37 CFR 1.116 to the outstanding final rejection. The response includes an amendment to at least one independent claim, and the amendment does not broaden the scope of the independent claim in any aspect.
4. This certification and request for consideration under AFCP 2.0 is the only AFCP 2.0 certification and request filed in response to the outstanding final rejection.
5. Applicant is willing and available to participate in any interview requested by the examiner concerning the present response.
6. This certification and request is being filed electronically using the Office's electronic filing system (EFS-Web).
7. Any fees that would be necessary consistent with current practice concerning responses after final rejection under 37 CFR 1.116, *e.g.*, extension of time fees, are being concurrently filed herewith. [There is no additional fee required to request consideration under AFCP 2.0.]
8. By filing this certification and request, applicant acknowledges the following:

- Reissue applications and reexamination proceedings are not eligible to participate in AFCP 2.0.
- The examiner will verify that the AFCP 2.0 submission is compliant, *i.e.*, that the requirements of the program have been met (see items 1 to 7 above). For compliant submissions:
    - The examiner will review the response under 37 CFR 1.116 to determine if additional search and/or consideration (i) is necessitated by the amendment and (ii) could be completed within the time allotted under AFCP 2.0. If additional search and/or consideration is required but cannot be completed within the allotted time, the examiner will process the submission consistent with current practice concerning responses after final rejection under 37 CFR 1.116, *e.g.*, by mailing an advisory action.
    - If the examiner determines that the amendment does not necessitate additional search and/or consideration, or if the examiner determines that additional search and/or consideration is required and could be completed within the allotted time, then the examiner will consider whether the amendment places the application in condition for allowance (after completing the additional search and/or consideration, if required). If the examiner determines that the amendment does not place the application in condition for allowance, then the examiner will contact the applicant and request an interview.
        - The interview will be conducted by the examiner, and if the examiner does not have negotiation authority, a primary examiner and/or supervisory patent examiner will also participate.
        - If the applicant declines the interview, or if the interview cannot be scheduled within ten (10) calendar days from the date that the examiner first contacts the applicant, then the examiner will proceed consistent with current practice concerning responses after final rejection under 37 CFR 1.116.

Signature: /Ian J.S. Lodovice, Reg. No. 59,749/	Date: May 27, 2015
Name (Print/Typed): Ian J. Lodovice	Practitioner Registration No. 59,749

***Note**: This form must be signed in accordance with 37 CFR 1.33. See 37 CFR 1.4(d) for signature requirements and certifications. Submit multiple forms if more than one signature is required, see below*.*

☒ * Total of 1 forms are submitted.

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IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

First Named Inventor	:	Brian K. Maples	Art Unit	:	1637
Serial No.	:	14/067,620	Examiner	:	Angela Marie Bertagna
Filed	:	October 30, 2013	Conf. No.	:	4288
Title	:	NICKING AND EXTENSION AMPLIFICATION REACTION FOR THE EXPONENTIAL AMPLIFICATION OF NUCLEIC ACIDS			

MAIL STOP AF
Commissioner for Patents
P.O. Box 1450
Alexandria, VA 22313-1450

AMENDMENT IN REPLY TO FINAL ACTION OF FEBRUARY 27, 2015

The present amendment is accompanied by a Certification and Request for Consideration Under the After Final Consideration Pilot Program 2.0 (AFCP 2.0).

Please amend the above-identified application as follows.

Amendments to the Claims:

This listing of claims replaces all prior versions and listings of claims in the application:

1. - 66. (Canceled)

67. (Currently Amended) A method of amplifying a target polynucleotide sequence of a target nucleic acid present in a sample obtained from an animal, the method comprising:

(a) preparing, without first subjecting the target nucleic acid to a denaturation step associated with amplification of the target polynucleotide sequence, a mixture comprising:

(i) the target nucleic acid comprising the target polynucleotide sequence,

(ii) a polymerase,

(iii) a nicking enzyme,

(iv) a first oligonucleotide comprising a nicking site and a nicking enzyme binding site, and

(v) a second oligonucleotide comprising a nicking site and a nicking enzyme binding site, and

(b) subjecting the mixture to essentially isothermal conditions to amplify the target polynucleotide sequence,

wherein amplification of the target polynucleotide sequence is performed at a constant temperature for about 1 to 12 minutes.

68. (Previously Presented) The method of claim 67, wherein the target polynucleotide sequence is amplified from steps comprising:

(a) forming a first duplex comprising the target polynucleotide sequence and the first oligonucleotide;

(b) extending, using the polymerase, the first oligonucleotide along the target polynucleotide sequence to form an extended first oligonucleotide comprising a sequence complementary to the second oligonucleotide;

(c) forming a second duplex comprising the second oligonucleotide and the extended first oligonucleotide;

(d) extending, using the polymerase, the second oligonucleotide along the extended first oligonucleotide to form a third duplex comprising an extended second oligonucleotide comprising a sequence complementary to the first oligonucleotide and a first double-stranded nicking enzyme binding site;

(e) nicking, with the nicking enzyme, the first nicking site on the third duplex to produce a fourth duplex comprising the extended second oligonucleotide and a fragment of the extended first oligonucleotide; and

(f) extending, using the polymerase, the fragment of the extended first oligonucleotide along the extended second oligonucleotide of the fourth duplex to produce a double-stranded nucleic acid product and a second double-stranded nicking enzyme binding site.

69. (Previously Presented) The method of claim 68, wherein the double-stranded nucleic acid product comprises:

i) a first strand and a second strand, wherein the first strand comprises a first polynucleotide sequence corresponding to the target polynucleotide sequence and the second strand comprises a second polynucleotide sequence complementary to the target polynucleotide sequence, and

ii) first and second double-stranded nicking sites spaced apart by the target polynucleotide sequence.

70. (Previously Presented) The method of claim 68, further comprising the steps of:

a) nicking, using the nicking enzyme, the first nicking site of the double-stranded nucleic acid product to produce a fifth duplex comprising a first polynucleotide sequence corresponding to the target polynucleotide sequence and a fragment of the first oligonucleotide, and nicking, using the nicking enzyme, the second nicking site of the double-stranded nucleic acid product to produce a sixth duplex comprising a second polynucleotide sequence complementary to the target polynucleotide sequence and a fragment of the second oligonucleotide;

b) extending, using the polymerase, the fragment of the first oligonucleotide along the first polynucleotide sequence of the fifth duplex to produce a first double stranded product comprising a copy of the nicking site and a copy of the first polynucleotide sequence and extending, using the polymerase, the fragment of the second oligonucleotide along the second polynucleotide sequence of the sixth duplex to produce a second double stranded product comprising a copy of the nicking site and a copy of the second polynucleotide sequence; and

c) nicking, using the nicking enzyme, the copy of the nicking site of the first double stranded product to release a copy of the first polynucleotide sequence and nicking, using the nicking enzyme, the copy of the nicking site of the second double stranded product to release a copy of the second polynucleotide sequence.

71. (Previously Presented) The method of claim 67, wherein the animal is a human.

72. (Previously Presented) The method of claim 67, wherein the target nucleic acid is obtained from an animal pathogen.

73. (Previously Presented) The method of claim 72, wherein the animal pathogen is a single-stranded DNA virus, double-stranded DNA virus, or single-stranded RNA virus.

74. (Withdrawn) The method of claim 72, wherein the animal pathogen is a bacterium.

75. (Withdrawn) The method of claim 72, wherein the animal pathogen contains spores and the target polynucleotide is amplified from the spores without the need for lysis of the spores.

76. (Previously Presented) The method of claim 67, wherein the sample obtained from an animal is obtained from the blood, bone marrow, mucus, lymph, hard tissues, biopsies, sputum, saliva, tears, faeces or urine of the animal.

77. (Previously Presented) The method of claim 76, wherein the sample obtained from an animal is obtained from the mucus, sputum, or saliva of the animal.

78. (Withdrawn) The method of claim 67, wherein the target nucleic acid is double-stranded DNA.

79. (Withdrawn) The method of claim 67, wherein the target nucleic acid is single-stranded DNA.

80. (Previously Presented) The method of claim 67, wherein the target nucleic acid is RNA.

81. (Withdrawn) The method of claim 67, wherein the target nucleic acid is selected from the group consisting of genomic DNA, plasmid DNA, viral DNA, mitochondrial DNA, cDNA, synthetic double-stranded DNA and synthetic single-stranded DNA.

82. (Withdrawn) The method of claim 81, wherein the target nucleic acid is genomic DNA.

83. (Previously Presented) The method of claim 67, wherein the target nucleic acid is viral DNA or viral RNA.

84. (Canceled)

85. (Previously Presented) The method of claim 67, wherein the nicking enzyme is Nt.BstNBI.

86. (Previously Presented) The method of claim 67, wherein the nicking enzyme does not nick within the target polynucleotide sequence.

87. (Previously Presented) The method of claim 67, wherein amplification of the target polynucleotide sequence is performed without the use of temperature cycling.

88. (Previously Presented) The method of claim 67, wherein amplification of the target polynucleotide sequence is performed at about 55°C-59°C.

89. (Canceled)

90. (Previously Presented) The method of claim 68, which is performed at a temperature higher than the melting temperature of the first oligonucleotide/target polynucleotide sequence complex.

91. (Previously Presented) The method of claim 67, further comprising detecting amplification product.

92. (Previously Presented) The method of claim 91, wherein the amplification product is detected by a detection method selected from the group consisting of gel electrophoresis, mass spectrometry, fluorescence, intercalating dye detection, fluorescence resonance energy transfer (FRET), molecular beacon detection, surface capture, capillary electrophoresis, incorporation of labeled nucleotides to allow detection by capture, fluorescence polarization, and lateral flow capture, or a combination thereof.

93. (Canceled)

94. (Canceled)

95. (Withdrawn, Currently Amended) A method of amplifying a target polynucleotide sequence of genomic DNA present in a sample obtained from an animal, the method comprising:

(a) preparing, without first subjecting the genomic DNA to a denaturation step associated with amplification of the target polynucleotide sequence, a mixture comprising:

(i) the genomic DNA comprising the target polynucleotide sequence,

(ii) a polymerase,

(iii) a nicking enzyme,

(iv) a first oligonucleotide comprising a nicking site and a nicking enzyme binding site, and

(v) a second oligonucleotide comprising a nicking site and a nicking enzyme binding site, and

b) subjecting the mixture to essentially isothermal conditions to amplify the target polynucleotide sequence,

wherein amplification of the target polynucleotide sequence is performed at a constant temperature for about 1 to 12 minutes.

96. (New) The method of claim 67, wherein the target polynucleotide sequence is amplified about 1E+8-fold.

97. (New) The method of claim 67, wherein the target polynucleotide sequence is amplified about 3E+9-fold.

98. (New) The method of claim 67, wherein the target polynucleotide sequence is amplified about 7E+10-fold.

99. (New) The method of claim 67, wherein amplification of the target polynucleotide sequence is performed at a constant temperature for about 1 to 10 minutes.

100. (New) The method of claim 67, wherein amplification of the target polynucleotide sequence is performed at a constant temperature for about 1 to 8 minutes.

101. (New) The method of claim 67, wherein amplification of the target polynucleotide sequence is performed at a constant temperature for about 1 to 5 minutes.

102. (New) The method of claim 67, wherein amplification of the target polynucleotide sequence is performed at a constant temperature for about 1 to 2.5 minutes.

103. (New) The method of claim 67, wherein amplification of the target polynucleotide sequence is performed at a constant temperature for about 2.5 to 5 minutes.

104. (New) The method of claim 67, wherein amplification of the target polynucleotide sequence is performed at a constant temperature for about 2.5 to 8 minutes.

105. (New) The method of claim 67, wherein amplification of the target polynucleotide sequence is performed at a constant temperature for about 2.5 to 10 minutes.

106. (New) A method of amplifying a target polynucleotide sequence of a target nucleic acid present in a sample obtained from an animal, the method comprising:

(a) preparing, without first subjecting the target nucleic acid to a denaturation step associated with amplification of the target polynucleotide sequence, a mixture comprising:

(i) the target nucleic acid comprising the target polynucleotide sequence,

(ii) a polymerase,

(iii) a nicking enzyme,

(iv) a first oligonucleotide comprising a nicking site and a nicking enzyme binding site, and

(v) a second oligonucleotide comprising a nicking site and a nicking enzyme binding site, and

(b) subjecting the mixture to essentially isothermal conditions to amplify the target polynucleotide sequence,

wherein the target polynucleotide sequence is amplified about 1E+8-fold in about 1 to 12 minutes.

REMARKS

Upon entry of the above amendment, claims 67-83, 85-88, 90-92 and 95-106 will be pending. Claims 1-66, 84 and 93-94 were previously canceled, and claim 89 is newly canceled. Claims 74-75, 78-79, 81-82 and 95 were previously withdrawn, so are not currently under examination. Claims 67 (and withdrawn claim 95) have been amended and new claims 96-106 have been added. Most of the amendments are simply to clarify scope and are supported throughout the specification.

For example, the amendment to claims 67 and 95 find support in Example 11. Claims 96-105 derive support from the specification at page 28, lines 4-5; and Examples 10-11. Claim 106 derives support from claim 67; and Examples 10-11.

No new matter has been introduced by these amendments. Reconsideration and allowance of the claims are respectfully requested in view of the above amendments and the following remarks.

Interview Request

Applicants respectfully request a telephonic interview to take place after the Examiner has reviewed these amendments and remarks, unless all the claims are then deemed allowable. So that the interview can take place at that point, and prior to preparation of a new Office Action (should that be deemed necessary), Applicants would be grateful if the Examiner would contact Applicants' representative Ian Lodovice at telephone number 617-956-5972 at the Examiner's convenience, to arrange an appropriate time for the interview.

Claim Rejection Under 35 USC § 112, First Paragraph (New Matter)

Claims 67-73, 76, 77, 80, 83 and 85-92 stand rejected under the first paragraph of 35 U.S.C. § 112 for allegedly failing to comply with the written description requirement. Specifically, the Office Action contends that Applicant's disclosure does not sufficiently provide support for "any type of denaturation step associated with amplification during the mixture preparation step." This contention is respectfully traversed.

When a disclosure describes a claimed invention in a manner that permits one skilled in the art to reasonably conclude that the inventor possessed the claimed invention the written description requirement is satisfied. (MPEP §2163). This possession may be shown in any number of ways and an Applicant need not describe every claim feature exactly because there is no *in haec verba* requirement. (MPEP § 2163). Rather, to satisfy the written description requirement, all that is required is "reasonable clarity." (MPEP § 2163.02). What is conventional or well known to one of ordinary skill in the art need not be disclosed in detail. See *Hybritech Inc. v. Monoclonal Antibodies, Inc.*, 802 F.2d at 1384, 231 USPQ at 94. See also *Capon v. Eshhar*, 418 F.3d 1349, 1357, 76 USPQ2d 1078, 1085 (Fed. Cir. 2005) ("The 'written description' requirement must be applied in the context of the particular invention and the state of the knowledge.... As each field evolves, the balance also evolves between what is known and what is added by each inventive contribution."). If a skilled artisan would have understood the inventor to be in possession of the claimed invention at the time of filing, even if every nuance of the claims is not explicitly described in the specification, then the adequate description requirement is met. See, e.g., *Vas-Cath*, 935 F.2d at 1563, 19 USPQ2d at 1116; *Martin v. Johnson*, 454 F.2d 746, 751, 172 USPQ 391, 395 (CCPA 1972) (stating "the description need not be *in ipsis verbis* [i.e., "in the same words"] to be sufficient").

Nucleic acid denaturation refers the formation of two free single-stranded nucleic acids from a duplex. It was well known to the skilled artisan that, at the time of filing, methods for the denaturation of nucleic acids were limited to a small and finite genus conventional methods. These conventional methods were: heat, chemical denaturants, enzymatic denaturants, and extremes of pH. Given this knowledge, one of ordinary skill in the art would understand that Applicant's disclosure provides adequate support for any type of denaturation step associated with amplification of the target polynucleotide sequence. One of ordinary skill would also understand that Applicants describe "an initial heat denaturation step" (see, for example, page 4, lines 16-29) as one example from what is known to be a limited genus of convention al methods, without intending to be limited or bound by this example or implying that denaturing by heat is a critical or essential element. Nowhere in the specification does it describe denaturing by heat as an essential or critical element of the claims. Rather, one of ordinary skill reading the disclosure would understand that the lack of denaturation by *any* condition or agent is required.

In view of the foregoing, Applicant respectfully submits that ordinarily skilled artisans would reasonably conclude that Applicant has adequately described the claimed methods. Reconsideration and withdrawal of the rejection of under 35 U.S.C. 112, first paragraph is requested.

Claim Rejection Under 35 USC § 103

Claims 67-73, 76, 77, 80, 83, and 85-92 stand rejected under pre-AIA 35 U.S.C. 103(a) as being unpatentable over Wick et al. (US 6,063,604)("Wick") in view of Kong et al. (US 6,191,267)("Kong") and further in view of Yao et al. (US 2009/0092967)("Yao"). As none of the references cited by the Office, considered along or in combination disclose each and every feature of amended claim 67, Applicant respectfully traverses.

As an initial matter, and as acknowledged by the Office, the Applicant maintains that neither Wick, nor Kong teach a method of amplifying a target polynucleotide sequence of a target nucleic acid in a sample, without first subjecting the target nucleic acid to a denaturation step associated with amplification of the target polynucleotide sequence, as required by the present claims. (See, e.g., Office Action at page 7-8). Moreover, the Applicant does not concede that there is a teaching, suggestion, or motivation to combine Wick and Kong in the manner indicated by the Examiner. And, even if there was motivation to combine Wick and Kong (and Applicant submits there is not), there is no reasonable expectation of success that the disparate teachings of the two references could even be successfully combined to arrive at the claimed invention.

Without conceding the appropriateness of the present rejection, and solely in the interest of advancing prosecution, claim 67 has been amended to require that "amplification of the target polynucleotide sequence is performed at a constant temperature for about 1 to 12 minutes." Thus, as amended, claim 67 requires preparing a mixture comprising a target nucleic acid and a nicking enzyme, without first subjecting the target nucleic acid to a denaturation step associated with amplification of the target polynucleotide sequence, and, after preparation of the mixture, the mixture is subjected to essentially isothermal conditions to amplify the target polynucleotide sequence, wherein amplification of the target polynucleotide sequence is performed at a constant temperature for about 1 to 12 minutes.

In addition to the reasons stated above, both Wick and Kong also fail to disclose a method as claimed, wherein amplification of the target polynucleotide sequence is performed at a constant temperature for about 1 to 12 minutes. In each of Wick's examples, samples were incubated for at least 30 minutes. (See, for example, Wick, col. 29, lines 1-10; Wick, col. 31, lines 5-16; Wick, col. 32, lines 19-26)

Kong fails to remedy the deficiencies of Wick. As noted by the Examiner, "Kong teaches that isothermal amplification reactions comprising strand displacement and used of a nicking enzyme may be completed in 20-60 minutes (column 14, lines 57-60)." (Office Action at page 8). Kong does not teach any examples where amplification of a target nucleic acid is sequence is performed at a constant temperature for about 1 to 12 minutes.

Yao also fails to remedy the deficiencies of Wick and Yao. As in Wick, in each of Yao's examples, samples were incubated for at least 30 minutes. (See, for example, Yao paragraphs [0112], [0115] and [0016]). As with Wick and Kong, Yao does not teach any examples where amplification of a target nucleic acid is sequence is performed at a constant temperature for about 1 to 12 minutes

For at least these reasons, independent claim 67 is patentable in view of the combination of Wick, Kong and Yao. Because independent claim 67 is patentable over Wick, Kong and Yao, dependent claims 68-73, 76, 77, 80, 83, and 85-88 and 90-92 are also patentable over Wick, Kong and Yao. For at least these reasons, Applicant requests reconsideration and withdrawal of this rejection.

Double Patenting

Claims 67-73, 76, 77, 80, 83, and 85-92 stand provisionally rejected on the ground of nonstatutory double patenting as being allegedly unpatentable over claims 67-69, 74-76, 79, 80, 83, 85, and 87-94 of copending Application No. 14/067,623.

Claims 67-73, 76, 77, 80, 83, and 85-92 stand provisionally rejected on the ground of nonstatutory double patenting as being allegedly unpatentable over claims 1-9, 12, 14-17, 19-41, and 44- 46 of copending Application No. 12/173,020.

Claims 67-73, 76, 77, 80, 83, 86-88, and 90-92 stand provisionally rejected on the ground of nonstatutory double patenting as being allegedly unpatentable over claims 125-130 of copending Application No. 11/778,018 in view of Wick.

Claims 85 and 89 are provisionally rejected stand provisionally rejected on the ground of nonstatutory double patenting as being allegedly unpatentable over claims 125-130 of copending Application No. 11/778,018 in view of Wick and further in view of Kong .

Applicant does not concede that the rejections above are appropriate. Further, as the applications cited above are all currently pending, Applicant requests that the rejections be held in abeyance pending the identification of allowable subject matter.

CONCLUSION

In light of the arguments made herein, applicants submit that the pending claims are patentable and request early and favorable action thereon. If any issues remain, the Examiner is asked to telephone the Applicants' representative Ian Lodovice at 617-956-5972 to arrange a time for an interview.

Applicants do not concede any positions of the Office that are not expressly addressed above, nor do applicants concede that there are not other good reasons for patentability of the presented claims or other claims.

This response is being filed with a Certification and Request for Consideration Under the After Final Consideration Pilot Program 2.0. Please apply any necessary charges or credits to Deposit Account 06-1050, referencing the above attorney docket number.

Respectfully submitted,

Date: May 27, 2015

/Ian J.S. Lodovice, Reg. No. 59,749/
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23363840.doc

Electronic Acknowledgement Receipt

EFS ID:	22462765
Application Number:	14067620
International Application Number:	
Confirmation Number:	4288
Title of Invention:	Nicking and Extension Amplification Reaction for the Exponential Amplification of Nucleic Acids
First Named Inventor/Applicant Name:	Brian K. Maples
Customer Number:	26161
Filer:	Ian J.S. Lodovice/Mary Florczak
Filer Authorized By:	Ian J.S. Lodovice
Attorney Docket Number:	30171-0025002 / ITI-001
Receipt Date:	27-MAY-2015
Filing Date:	30-OCT-2013
Time Stamp:	16:56:24
Application Type:	Utility under 35 USC 111(a)

Payment information:

Submitted with Payment	no

File Listing:

Document Number	Document Description	File Name	File Size(Bytes)/ Message Digest	Multi Part /.zip	Pages (if appl.)
1	After Final Consideration Program Request	30171AFCP.pdf	152750 ca41752e9b4bb3c7f0f5f97cf9ec61bade879d48	no	1

Warnings:

Information:

2		30171Response.pdf	150844 f051142f9fe03c145736c3ed4f7f7eab532bf814	yes	14

Multipart Description/PDF files in .zip description		
Document Description	**Start**	**End**
Response After Final Action	1	1
Claims	2	8
Applicant Arguments/Remarks Made in an Amendment	9	14

Warnings:

Information:

Total Files Size (in bytes):	303594

This Acknowledgement Receipt evidences receipt on the noted date by the USPTO of the indicated documents, characterized by the applicant, and including page counts, where applicable. It serves as evidence of receipt similar to a Post Card, as described in MPEP 503.

<u>New Applications Under 35 U.S.C. 111</u>

If a new application is being filed and the application includes the necessary components for a filing date (see 37 CFR 1.53(b)-(d) and MPEP 506), a Filing Receipt (37 CFR 1.54) will be issued in due course and the date shown on this Acknowledgement Receipt will establish the filing date of the application.

<u>National Stage of an International Application under 35 U.S.C. 371</u>

If a timely submission to enter the national stage of an international application is compliant with the conditions of 35 U.S.C. 371 and other applicable requirements a Form PCT/DO/EO/903 indicating acceptance of the application as a national stage submission under 35 U.S.C. 371 will be issued in addition to the Filing Receipt, in due course.

<u>New International Application Filed with the USPTO as a Receiving Office</u>

If a new international application is being filed and the international application includes the necessary components for an international filing date (see PCT Article 11 and MPEP 1810), a Notification of the International Application Number and of the International Filing Date (Form PCT/RO/105) will be issued in due course, subject to prescriptions concerning national security, and the date shown on this Acknowledgement Receipt will establish the international filing date of the application.

PTO/SB/06 (09-11)
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PATENT APPLICATION FEE DETERMINATION RECORD Substitute for Form PTO-875	Application or Docket Number 14/067,620	Filing Date 10/30/2013	☐ To be Mailed

ENTITY: ☒ LARGE ☐ SMALL ☐ MICRO

APPLICATION AS FILED – PART I

FOR	NUMBER FILED (Column 1)	NUMBER EXTRA (Column 2)	RATE ($)	FEE ($)
☐ BASIC FEE (37 CFR 1.16(a), (b), or (c))	N/A	N/A	N/A	
☐ SEARCH FEE (37 CFR 1.16(k), (i), or (m))	N/A	N/A	N/A	
☐ EXAMINATION FEE (37 CFR 1.16(o), (p), or (q))	N/A	N/A	N/A	
TOTAL CLAIMS (37 CFR 1.16(i))	minus 20 =	*	X $ =	
INDEPENDENT CLAIMS (37 CFR 1.16(h))	minus 3 =	*	X $ =	
☐ APPLICATION SIZE FEE (37 CFR 1.16(s))	If the specification and drawings exceed 100 sheets of paper, the application size fee due is $310 ($155 for small entity) for each additional 50 sheets or fraction thereof. See 35 U.S.C. 41(a)(1)(G) and 37 CFR 1.16(s).			
☐ MULTIPLE DEPENDENT CLAIM PRESENT (37 CFR 1.16(j))				
* If the difference in column 1 is less than zero, enter "0" in column 2.			TOTAL	

APPLICATION AS AMENDED – PART II

AMENDMENT	05/27/2015	CLAIMS REMAINING AFTER AMENDMENT (Column 1)		HIGHEST NUMBER PREVIOUSLY PAID FOR (Column 2)	PRESENT EXTRA (Column 3)	RATE ($)	ADDITIONAL FEE ($)
	Total (37 CFR 1.16(i))	* 36	Minus	** 29	= 7	x $80 =	560
	Independent (37 CFR 1.16(h))	* 3	Minus	***3	= 0	x $420 =	0
	☐ Application Size Fee (37 CFR 1.16(s))						
	☐ FIRST PRESENTATION OF MULTIPLE DEPENDENT CLAIM (37 CFR 1.16(j))						
						TOTAL ADD'L FEE	**560**

AMENDMENT		CLAIMS REMAINING AFTER AMENDMENT (Column 1)		HIGHEST NUMBER PREVIOUSLY PAID FOR (Column 2)	PRESENT EXTRA (Column 3)	RATE ($)	ADDITIONAL FEE ($)
	Total (37 CFR 1.16(i))	*	Minus	**	=	X $ =	
	Independent (37 CFR 1.16(h))	*	Minus	***	=	X $ =	
	☐ Application Size Fee (37 CFR 1.16(s))						
	☐ FIRST PRESENTATION OF MULTIPLE DEPENDENT CLAIM (37 CFR 1.16(j))						
						TOTAL ADD'L FEE	

* If the entry in column 1 is less than the entry in column 2, write "0" in column 3.
** If the "Highest Number Previously Paid For" IN THIS SPACE is less than 20, enter "20".
*** If the "Highest Number Previously Paid For" IN THIS SPACE is less than 3, enter "3".
The "Highest Number Previously Paid For" (Total or Independent) is the highest number found in the appropriate box in column 1.

LIE
/YOLANDA CHADWICK/

This collection of information is required by 37 CFR 1.16. The information is required to obtain or retain a benefit by the public which is to file (and by the USPTO to process) an application. Confidentiality is governed by 35 U.S.C. 122 and 37 CFR 1.14. This collection is estimated to take 12 minutes to complete, including gathering, preparing, and submitting the completed application form to the USPTO. Time will vary depending upon the individual case. Any comments on the amount of time you require to complete this form and/or suggestions for reducing this burden, should be sent to the Chief Information Officer, U.S. Patent and Trademark Office, U.S. Department of Commerce, P.O. Box 1450, Alexandria, VA 22313-1450. DO NOT SEND FEES OR COMPLETED FORMS TO THIS ADDRESS. **SEND TO: Commissioner for Patents, P.O. Box 1450, Alexandria, VA 22313-1450.**
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APPLICATION NO.	FILING DATE	FIRST NAMED INVENTOR	ATTORNEY DOCKET NO.	CONFIRMATION NO.
14/067,620	10/30/2013	Brian K. Maples	30171-0025002 / ITI-001	4288

26161 7590 02/27/2015
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EXAMINER	
BERTAGNA, ANGELA MARIE	
ART UNIT	**PAPER NUMBER**
1637	
NOTIFICATION DATE	**DELIVERY MODE**
02/27/2015	ELECTRONIC

Please find below and/or attached an Office communication concerning this application or proceeding.

The time period for reply, if any, is set in the attached communication.

Notice of the Office communication was sent electronically on above-indicated "Notification Date" to the following e-mail address(es):

PATDOCTC@fr.com

PTOL-90A (Rev. 04/07)

Office Action Summary	Application No. 14/067,620	Applicant(s) MAPLES ET AL.	
	Examiner Angela M. Bertagna	Art Unit 1637	AIA (First Inventor to File) Status No

-- The MAILING DATE of this communication appears on the cover sheet with the correspondence address --

Period for Reply

A SHORTENED STATUTORY PERIOD FOR REPLY IS SET TO EXPIRE *3* MONTHS FROM THE MAILING DATE OF THIS COMMUNICATION.

- Extensions of time may be available under the provisions of 37 CFR 1.136(a). In no event, however, may a reply be timely filed after SIX (6) MONTHS from the mailing date of this communication.
- If NO period for reply is specified above, the maximum statutory period will apply and will expire SIX (6) MONTHS from the mailing date of this communication.
- Failure to reply within the set or extended period for reply will, by statute, cause the application to become ABANDONED (35 U.S.C. § 133). Any reply received by the Office later than three months after the mailing date of this communication, even if timely filed, may reduce any earned patent term adjustment. See 37 CFR 1.704(b).

Status

1)☒ Responsive to communication(s) filed on *1/9/15*.
☐ A declaration(s)/affidavit(s) under **37 CFR 1.130(b)** was/were filed on _____.

2a)☒ This action is **FINAL**. 2b)☐ This action is non-final.

3)☐ An election was made by the applicant in response to a restriction requirement set forth during the interview on _____; the restriction requirement and election have been incorporated into this action.

4)☐ Since this application is in condition for allowance except for formal matters, prosecution as to the merits is closed in accordance with the practice under *Ex parte Quayle*, 1935 C.D. 11, 453 O.G. 213.

Disposition of Claims*

5)☒ Claim(s) *67-83,85-92 and 95* is/are pending in the application.
5a) Of the above claim(s) *74,75,78,79,81,82 and 95* is/are withdrawn from consideration.

6)☐ Claim(s) _____ is/are allowed.

7)☒ Claim(s) *67-73,76,77,80,83 and 85-92* is/are rejected.

8)☐ Claim(s) _____ is/are objected to.

9)☐ Claim(s) _____ are subject to restriction and/or election requirement.

* If any claims have been determined allowable, you may be eligible to benefit from the **Patent Prosecution Highway** program at a participating intellectual property office for the corresponding application. For more information, please see http://www.uspto.gov/patents/init_events/pph/index.jsp or send an inquiry to PPHfeedback@uspto.gov.

Application Papers

10)☐ The specification is objected to by the Examiner.

11)☒ The drawing(s) filed on *1/9/15* is/are: a)☒ accepted or b)☐ objected to by the Examiner.
Applicant may not request that any objection to the drawing(s) be held in abeyance. See 37 CFR 1.85(a).
Replacement drawing sheet(s) including the correction is required if the drawing(s) is objected to. See 37 CFR 1.121(d).

Priority under 35 U.S.C. § 119

12)☐ Acknowledgment is made of a claim for foreign priority under 35 U.S.C. § 119(a)-(d) or (f).

Certified copies:

a)☐ All b)☐ Some** c)☐ None of the:

1.☐ Certified copies of the priority documents have been received.

2.☐ Certified copies of the priority documents have been received in Application No. _____.

3.☐ Copies of the certified copies of the priority documents have been received in this National Stage application from the International Bureau (PCT Rule 17.2(a)).

** See the attached detailed Office action for a list of the certified copies not received.

Attachment(s)

1) ☒ Notice of References Cited (PTO-892)

2) ☒ Information Disclosure Statement(s) (PTO/SB/08a and/or PTO/SB/08b)
Paper No(s)/Mail Date *12/5/14; 1/9/15; 2/2/15*.

3) ☐ Interview Summary (PTO-413)
Paper No(s)/Mail Date. _____ .

4) ☐ Other: _____.

DETAILED ACTION

Notice of Pre-AIA or AIA Status

1. The present application is being examined under the pre-AIA first to invent provisions.

Status of the Application

2. Applicant's response filed on January 9, 2015 is acknowledged. Claims 67-83, 85-92, and 95 are pending. Claims 67-73, 76, 77, 80, 83, and 85-92 are under examination. Claims 74, 75, 78, 79, 81, 82, and 95 are withdrawn as being drawn to a non-elected species.

The following includes new grounds of rejection necessitated by Applicant's amendments to the claims. Any previously made rejections or objections not reiterated below have been withdrawn.

Response to Arguments

3. Applicant's arguments filed on January 9, 2015 have been fully considered.

Objection to the Drawings

Applicant argues that the objection should be withdrawn in view of the submission of replacement drawings (page 9). This argument was persuasive. The objection has been withdrawn. The replacement drawings submitted on January 9, 2015 are acceptable.

Objection to the Specification

As noted by Applicant on page 9, claim 93 has been canceled. The objection to the specification for failing to provide proper antecedent basis for the subject matter of this claim has been withdrawn accordingly.

Rejection under pre-AIA 35 U.S.C. 112, second paragraph

Applicant argues that the rejection of claims 76, 77, 84, and 87-94 should be withdrawn in view of the claim amendments (page 9). This argument was persuasive. The rejection has been withdrawn.

Rejection of claims 67, 71-73, 76, 80, 83, 85, and 86 under pre-AIA 35 U.S.C. 102(b) as being anticipated by Van Ness et al. (WO 2003/008642 A1)

Applicant's arguments, see pages 10-12, have been fully considered and are persuasive. The rejection has been withdrawn.

Rejection of claims 67-73, 76, 77, 80, and 83-94 under pre-AIA 35 U.S.C. 103(a) as being unpatentable over Wick et al. (US 6,063,604) in view of Kong et al. (US 6,191,267)

Applicant argues that the rejection should be withdrawn because the cited references do not teach or suggest conducting an isothermal amplification reaction without an initial denaturation step as required by the amended claims (page 12). This argument was persuasive. The rejection has been withdrawn. The new rejection set forth below cites an additional reference to address this requirement of the amended claims.

Applicant additionally argues, see pages 12-13, that there is no motivation to combine the references because the disclosure of Kong is limited to amplification of a double-stranded DNA target using four primers, whereas Wick describes amplification of single-stranded RNA targets using only two primers. Applicant argues that the ordinary artisan would have had neither a motivation nor a reasonable expectation of success in combining such different references.

This argument applies to the new rejection set forth below. It was not persuasive because the Wick and Kong references are not, in fact, so very different from each other. The working

example of Kong describes amplification of a double-stranded DNA target using the disclosed Nt.BstNBI enzyme and a SDA method using four primers, two of which contain a nicking recognition and cleavage site for the Nt.BstNBI enzyme and two of which are outer (bumper) primers lacking a nicking recognition or cleavage site, but the reference in no way suggests that the disclosed Nt.BstNBI enzyme is unsuitable for use in SDA methods conducted without bumper primers or that a double-stranded DNA target is required. Rather, the teachings of the reference indicate that the disclosed Nt.BstNBI enzyme is generally useful in strand displacement amplification reactions since its use eliminates the need to use modified nucleotides, such as those used in the method of Wick. In view of the above, the ordinary artisan would have had a motivation and reasonable expectation of success in practicing the method of Wick using the Nt.BstNBI enzyme described by Kong.

Double Patenting

Applicant requests that the issue be held in abeyance until allowable subject matter has been identified (page 13). The rejections have been maintained with modifications necessitated by amendment since they are still applicable.

Claim Rejections - 35 USC § 112 (New Matter)

4. The following is a quotation of the first paragraph of 35 U.S.C. 112(a):

> (a) IN GENERAL.—The specification shall contain a written description of the invention, and of the manner and process of making and using it, in such full, clear, concise, and exact terms as to enable any person skilled in the art to which it pertains, or with which it is most nearly connected, to make and use the same, and shall set forth the best mode contemplated by the inventor or joint inventor of carrying out the invention.

The following is a quotation of the first paragraph of pre-AIA 35 U.S.C. 112:

> The specification shall contain a written description of the invention, and of the manner and process of making and using it, in such full, clear, concise, and exact terms as to enable any person skilled in the

> art to which it pertains, or with which it is most nearly connected, to make and use the same, and shall set forth the best mode contemplated by the inventor of carrying out his invention.

Claims 67-73, 76, 77, 80, 83, and 85-92 are rejected under 35 U.S.C. 112(a) or 35 U.S.C. 112 (pre-AIA), first paragraph, as failing to comply with the written description requirement. The claim(s) contains subject matter which was not described in the specification in such a way as to reasonably convey to one skilled in the relevant art that the inventor or a joint inventor, or for pre-AIA the inventor(s), at the time the application was filed, had possession of the claimed invention. **This is a new matter rejection.**

Independent claim 67 has been amended to require the mixture to be prepared "without first subjecting the target nucleic acid to a denaturation step associated with amplification of the target polynucleotide sequence".

Applicant states that the limitation finds support at page 4, lines 6-29, Figures 1A-D, and throughout the examples (page 8 of the response).

The original disclosure, including the portions cited by Applicant, has been reviewed, but support was not found for the amendment to claim 67. The language of amended claim 67 encompasses omitting any type of denaturation step associated with amplification during the mixture preparation step, but the original disclosure only provides support for omitting a heat denaturation step (page 4, lines 16-29, for example). Accordingly, the amendment to claim 67 introduces new matter because the original disclosure does not provide support for the more generic description of denaturation recited in the claim.

Claim Rejections - 35 USC § 103

5. The following is a quotation of pre-AIA 35 U.S.C. 103(a) which forms the basis for all

obviousness rejections set forth in this Office action:

> (a) A patent may not be obtained though the invention is not identically disclosed or described as set forth in section 102 of this title, if the differences between the subject matter sought to be patented and the prior art are such that the subject matter as a whole would have been obvious at the time the invention was made to a person having ordinary skill in the art to which said subject matter pertains. Patentability shall not be negatived by the manner in which the invention was made.

This application currently names joint inventors. In considering patentability of the claims under pre-AIA 35 U.S.C. 103(a), the examiner presumes that the subject matter of the various claims was commonly owned at the time any inventions covered therein were made absent any evidence to the contrary. Applicant is advised of the obligation under 37 CFR 1.56 to point out the inventor and invention dates of each claim that was not commonly owned at the time a later invention was made in order for the examiner to consider the applicability of pre-AIA 35 U.S.C. 103(c) and potential pre-AIA 35 U.S.C. 102(e), (f) or (g) prior art under pre-AIA 35 U.S.C. 103(a).

6. Claims 67-73, 76, 77, 80, 83, and 85-92 are rejected under pre-AIA 35 U.S.C. 103(a) as being unpatentable over Wick et al. (US 6,063,604) in view of Kong et al. (US 6,191,267) and further in view of Yao et al. (US 2009/0092967 A1).

Regarding claims 67-73, 76, 77, 80, and 83, Wick teaches a method that comprises subjecting a mixture of the following components to an amplification reaction: (i) a target nucleic acid, (ii) a polymerase, (iii) a restriction endonuclease capable of nicking at a hemi-modified recognition site, and (iv) two oligonucleotides, each comprising a recognition sequence for the restriction enzyme of (iii) (see, for example, Figure 1, column 10, line 39 – column 11, line 67, column 13, lines 7-52, and columns 25-28 ("Segregation and/or Amplification Scheme"); see also the Examples at columns 28-31). In the amplification method of Wick, the

steps set forth in parts (a)-(f) of claim 68, parts (i) and (ii) of claim 69, and parts (a)-(c) of claim 70 occur (see Figures 1a-1b and the accompanying discussion at columns 25-28).The reference teaches that the target nucleic acid may be single-stranded viral RNA from an animal pathogen (see, for example, column 12, lines 17-21, 24-30, 34-38, and 62-66) and that samples containing the target nucleic acid may be isolated from tissue (e.g., liver, lung, kidney, and spleen), blood, tears, feces, urine, sputum, mucus, bone marrow, tissues or saliva samples obtained from humans suspected of having been in contact with the virus (see, for example, column 12, lines 24-30 and 34-38 as well as column 13, lines 53-63).

Regarding claim 86, Wick teaches that the nicking step does not result in nicking within the target polynucleotide sequence (see Figure 1b, for example).

Regarding claims 87 and 88, Wick teaches that the method is conducted isothermally at a temperatures within the claimed range (see, for example, column 13, lines 7-52, column 24, lines 33-67, and column 29, lines 2-8).

Regarding claims 91 and 92, Wick teaches detecting the amplification product using at least one of the required methods (see, for example, column 29, lines 11-44).

In the method of Wick, modified nucleotides (e.g., phosphorothioated nucleotides) are used to create a hemi-modified nucleic acid substrate for nicking (column 15, lines 17-53). As a result, the reference fails to teach the use of a nicking enzyme (e.g., Nt.BstNBI), as required by all of the claims. The reference also does not teach omitting an initial, pre-amplification denaturation step as required by all of the claims. Further, Wick does not teach that the amplification time is about 1-20 minutes as required by claim 89 or that the amplification

reaction is performed at a temperature that is higher than the melting temperature of the duplex formed between the first oligonucleotide and the target polynucleotide.

Kong describes methods for making and using Nt.BstNBI, which is a nicking enzyme (abstract and column 4, lines 21-50; see also Example 4 at column 14). The reference further teaches that the modified nucleotides typically used in strand displacement amplification are expensive and poorly incorporated by DNA polymerase, but that the use of a nicking enzyme eliminates the need for their use (column 2, lines 4-45). Further regarding claim 89, Kong teaches that isothermal amplification reactions comprising strand displacement and use of a nicking enzyme may be completed in 20-60 minutes (column 14, lines 57-60).

The method of Kong does not omit an initial, pre-amplification denaturation step.

Yao describes conducting strand displacement amplification reactions in the absence of an initial pre-amplification denaturation step (see, for example, paragraphs 18, 28-42, and Examples 1-2 on pages 13-14). The reference teaches that eliminating the conventional pre-amplification step results in a simpler, more efficient, and more flexible method (paragraph 18).

It would have been *prima facie* obvious for one of ordinary skill in the art at the time of the invention to conduct the isothermal amplification method of Wick using the Nt.BstNBI nicking enzyme described by Kong. The ordinary artisan would have been motivated to do so since Kong taught that using a nicking enzyme eliminates the need to use more expensive and poorly incorporated modified nucleotides in isothermal amplification reactions. The ordinary artisan would have had a reasonable expectation of success since Kong described methods for obtaining the enzyme (Examples 1-3 at columns 6-14) as well as a method for using it in an isothermal strand displacement amplification reaction (Example 4 at column 14). It also would

have been *prima facie* obvious for the ordinary artisan to omit the initial denaturation step when practicing the method suggested by the teachings of Wick and Kong. Motivation and a reasonable expectation of success are provided by Yao since the reference teaches that this step may be omitted to obtain a simpler, more efficient, and more flexible method (paragraph 18 and Examples 1-2 on pages 13-14).

Further regarding claim 89, it also would have been *prima facie* obvious for one of ordinary skill in the art at the time of the invention to conduct the amplification step in the method suggested by the teachings of Wick, Kong, and Yao for a time within the claimed range of about 1-20 minutes. The ordinary artisan would have been motivated to do so with a reasonable expectation of success in view of the teachings of Kong in Example 4, which indicate that isothermal amplification reactions comprising strand displacement and use of a nicking enzyme may be completed in 20-60 minutes (column 14, lines 57-60).

Further regarding claim 90, it also would have been *prima facie* obvious for one of ordinary skill in the art at the time of the invention to conduct the amplification step in the method suggested by the teachings of Wick, Kong, and Yao at a temperature that is higher than the melting temperature of the duplex formed between the first oligonucleotide and the target polynucleotide. The ordinary artisan would have recognized that the temperature at which the amplification reaction is conducted is a results-effective variable and, accordingly, would have been motivated to conduct routine experimentation to determine an optimal temperature with a reasonable expectation of success. As discussed in MPEP 2144.05, performing routine experimentation to optimize results-effective variables is *prima facie* obvious in the absence of secondary considerations. In this case, no evidence of unexpected results has been presented.

Thus, the methods of claims 67-73, 76, 77, 80, 83, and 85-92 are *prima facie* obvious over Wick in view of Kong and further in view of Yao.

Double Patenting

7. The nonstatutory double patenting rejection is based on a judicially created doctrine grounded in public policy (a policy reflected in the statute) so as to prevent the unjustified or improper timewise extension of the "right to exclude" granted by a patent and to prevent possible harassment by multiple assignees. A nonstatutory double patenting rejection is appropriate where the claims at issue are not identical, but at least one examined application claim is not patentably distinct from the reference claim(s) because the examined application claim is either anticipated by, or would have been obvious over, the reference claim(s). See, e.g., *In re Berg*, 140 F.3d 1428, 46 USPQ2d 1226 (Fed. Cir. 1998); *In re Goodman*, 11 F.3d 1046, 29 USPQ2d 2010 (Fed. Cir. 1993); *In re Longi*, 759 F.2d 887, 225 USPQ 645 (Fed. Cir. 1985); *In re Van Ornum*, 686 F.2d 937, 214 USPQ 761 (CCPA 1982); *In re Vogel*, 422 F.2d 438, 164 USPQ 619 (CCPA 1970); and *In re Thorington*, 418 F.2d 528, 163 USPQ 644 (CCPA 1969).

A timely filed terminal disclaimer in compliance with 37 CFR 1.321(c) or 1.321(d) may be used to overcome an actual or provisional rejection based on a nonstatutory double patenting ground provided the reference application or patent either is shown to be commonly owned with this application, or claims an invention made as a result of activities undertaken within the scope of a joint research agreement. A terminal disclaimer must be signed in compliance with 37 CFR 1.321(b).

The USPTO internet Web site contains terminal disclaimer forms which may be used. Please visit http://www.uspto.gov/forms/. The filing date of the application will determine what form should be used. A web-based eTerminal Disclaimer may be filled out completely online using web-screens. An eTerminal Disclaimer that meets all requirements is auto-processed and approved immediately upon submission. For more information about eTerminal Disclaimers, refer to http://www.uspto.gov/patents/process/file/efs/guidance/eTD-info-I.jsp.

8. Claims 67-73, 76, 77, 80, 83, and 85-92 are provisionally rejected on the ground of nonstatutory double patenting as being unpatentable over claims 67-69, 74-76, 79, 80, 83, 85, and 87-94 of copending Application No. 14/067,623. Although the claims at issue are not identical, they are not patentably distinct from each other because the claims of the '623 application overlap in scope with the claimed methods and recite all of their limitations.

This is a provisional nonstatutory double patenting rejection because the patentably indistinct claims have not in fact been patented.

9. Claims 67-73, 76, 77, 80, 83, and 85-92 are provisionally rejected on the ground of nonstatutory double patenting as being unpatentable over claims 1-9, 12, 14-17, 19-41, and 44-46 of copending Application No. 12/173,020.

Although the claims at issue are not identical, they are not patentably distinct from each other because the claims of the ‘020 application are also drawn to an isothermal amplification method omitting an initial denaturation step and comprising the use of nicking enzymes and primers having nicking enzyme recognition and cleavage sites (see, in particular, claims 1, 41,

42, 44, and 47). The claims of the '020 application also suggest the use of single-stranded viral RNA in a sample from an animal as the target polynucleotide (see, in particular, claims 9, 12, 14, 45, and 46). Further regarding the instant claim 77, mucus, saliva, and sputum were generally known in the art to be useful samples for nucleic acid amplification procedures. Accordingly, this claim is also not patentably distinct from the claims of the '020 application. Further regarding the instant claim 90, although the claims of the '020 application do not state that the amplification step is performed at a temperature that is higher than the melting temperature of the duplex formed between the first oligonucleotide template and the target polynucleotide, the ordinary artisan would have recognized that the temperature at which the amplification reaction is conducted is a results-effective variable and, accordingly, would have been motivated to conduct routine experimentation to determine an optimal temperature with a reasonable expectation of success. As discussed in MPEP 2144.05, performing routine experimentation to optimize results-effective variables is *prima facie* obvious in the absence of secondary considerations. In this case, no evidence of unexpected results has been presented. Thus, the instant claims 67-73, 76, 77, 80, 83, and 85-92 are not patentably distinct from claims 1-9, 12, 14-17, 19-41, and 44-46 of the ‘020 application.

This is a provisional nonstatutory double patenting rejection because the patentably indistinct claims have not in fact been patented.

10. Claims 67-73, 76, 77, 80, 83, 86-88, and 90-92 are provisionally rejected on the ground of nonstatutory double patenting as being unpatentable over claims 125-130 of copending Application No. 11/778,018 in view of Wick et al. (US 6,063,604).

The instant claims are drawn to method for isothermal amplification of a single-stranded viral RNA target that omits an initial denaturation step and comprises the use of nicking enzymes. Claims 125-130 of the ‘018 application are also drawn to a method for isothermal amplification of a single-stranded RNA target that comprises the use of nicking enzymes and omits an initial denaturation step. The claims of the ‘018 application disclose or suggest all of the limitations of the instant claims 67-73, 76, 77, 80, 83, 86-88, and 90-92, but they do not state that the RNA target is a single-stranded viral RNA contained in a sample obtained from an animal as required by the instant claims. The claims of the '018 application also do not state that the method further comprises detection of the amplification product as required by the instant claims 91 and 92. Further, the claims of the ‘018 application do not teach the requirements of the instant claim 90.

Wick teaches a method that comprises subjecting a mixture of the following components to an iosothermal amplification reaction: (i) a target nucleic acid, (ii) a polymerase, (iii) a restriction endonuclease capable of nicking at a hemi-modified recognition site, and (iv) two oligonucleotides, each comprising a recognition sequence for the restriction enzyme of (iii) (see, for example, Figure 1, column 10, line 39 – column 11, line 67, column 13, lines 7-52, and columns 25-28 (“Segregation and/or Amplification Scheme”); see also the Examples at columns 28-31). The reference teaches that the target nucleic acid may be single-stranded viral RNA from an animal pathogen (see, for example, column 12, lines 17-21, 24-30, 34-38, and 62-66) and that samples containing the target nucleic acid may be isolated from tissue (e.g., liver, lung, kidney, and spleen), blood, tears, feces, urine, sputum, mucus, bone marrow, tissues or saliva samples

obtained from humans suspected of having been in contact with the virus (see, for example, column 12, lines 24-30 and 34-38 as well as column 13, lines 53-63).

Regarding claims 91 and 92, Wick teaches detecting the amplification product using at least one of the required methods (see, for example, column 29, lines 11-44).

It would have been *prima facie* obvious for one of ordinary skill in the art at the time of the invention to practice the isothermal amplification method recited in the claims of the '018 application using a single-stranded viral RNA contained in a sample from an animal as the target nucleic acid. The ordinary artisan would have been motivated to do so with a reasonable expectation of success in view of the teachings of Wick, which indicate that such samples and targets may be used in a similar isothermal amplification method comprising nicking and strand displacement. The ordinary artisan also would have been motivated to detect the amplification product using a method described by Wick since detection of amplification products was routine and conventional in the art at the time of the invention. Finally, further regarding the instant claim 90, it would have been *prima facie* obvious for one of ordinary skill in the art at the time of the invention to conduct the amplification step at a temperature that is higher than the melting temperature of the duplex formed between the first oligonucleotide and the target polynucleotide. The ordinary artisan would have recognized that the temperature at which the amplification reaction is conducted is a results-effective variable and, accordingly, would have been motivated to conduct routine experimentation to determine an optimal temperature with a reasonable expectation of success. As discussed in MPEP 2144.05, performing routine experimentation to optimize results-effective variables is *prima facie* obvious in the absence of secondary considerations. In this case, no evidence of unexpected results has been presented.

Thus, the instant claims 67-73, 76, 77, 80, 83, 86-88, and 90-92 are not patentably distinct from claims 125-130 of the ‘018 application in view of Wick.

This is a provisional nonstatutory double patenting rejection because the patentably indistinct claims have not in fact been patented.

11. Claims 85 and 89 are provisionally rejected on the ground of nonstatutory double patenting as being unpatentable over claims 125-130 of copending Application No. 11/778,018 in view of Wick et al. (US 6,063,604) and further in view of Kong et al. (US 6,191,267).

As discussed above, the instant claims 67-73, 76, 77, 80, 83, 86-88, and 90-92 are not patentably distinct from claims 125-130 of the ‘018 application in view of Wick.

Neither Wick nor the claims of the ‘018 application teach conducting the amplification reaction for a time within the range set forth in claim 89 or the use of Nt.BstNBI as the nicking enzyme as required by the instant claim 85.

Kong describes methods for making and using Nt.BstNBI, which is a nicking enzyme suitable for use in isothermal strand displacement amplification methods comprising a nicking step (abstract and column 4, lines 21-50; see also Example 4 at column 14). The reference also teaches that isothermal amplification reactions comprising strand displacement and use of a nicking enzyme may be completed in 20-60 minutes (column 14, lines 57-60).

It would have been *prima facie* obvious for the ordinary artisan practicing the method suggested by the claims of the '018 application and Wick to select Nt.BstNBI as the nicking enzyme. The ordinary artisan would have been motivated to do so with a reasonable expectation of success since Kong taught that this nicking enzyme was suitable for use in isothermal strand

displacement amplification reactions comprising a nicking step and also eliminated the need to use expensive and poorly incorporated modified nucleotides. It also would have been *prima facie* obvious for the ordinary artisan practicing the method suggested by the claims of the '018 application and Wick to conduct the amplification step for a time within the claimed range of about 1-20 minutes. The ordinary artisan would have been motivated to do so with a reasonable expectation of success in view of the teachings of Kong in Example 4, which indicate that isothermal amplification reactions comprising strand displacement and use of a nicking enzyme may be completed in 20-60 minutes (column 14, lines 57-60).

Thus, the instant claims 85 and 89 are not patentably distinct from claims 125-130 of the '018 application in view of Wick and further in view of Kong.

This is a provisional nonstatutory double patenting rejection because the patentably indistinct claims have not in fact been patented.

Conclusion

12. No claims are currently allowable.

Applicant's amendment necessitated the new ground(s) of rejection presented in this Office action. Accordingly, **THIS ACTION IS MADE FINAL**. See MPEP § 706.07(a). Applicant is reminded of the extension of time policy as set forth in 37 CFR 1.136(a).

A shortened statutory period for reply to this final action is set to expire THREE MONTHS from the mailing date of this action. In the event a first reply is filed within TWO MONTHS of the mailing date of this final action and the advisory action is not mailed until after the end of the THREE-MONTH shortened statutory period, then the shortened statutory period

will expire on the date the advisory action is mailed, and any extension fee pursuant to 37 CFR 1.136(a) will be calculated from the mailing date of the advisory action. In no event, however, will the statutory period for reply expire later than SIX MONTHS from the date of this final action.

Any inquiry concerning this communication or earlier communications from the examiner should be directed to Angela M. Bertagna whose telephone number is (571)272-8291. The examiner can normally be reached on Monday-Friday, 9-5.

If attempts to reach the examiner by telephone are unsuccessful, the examiner's supervisor, Gary Benzion can be reached on (571)272-0782. The fax phone number for the organization where this application or proceeding is assigned is 571-273-8300.

Information regarding the status of an application may be obtained from the Patent Application Information Retrieval (PAIR) system. Status information for published applications may be obtained from either Private PAIR or Public PAIR. Status information for unpublished applications is available through Private PAIR only. For more information about the PAIR system, see http://pair-direct.uspto.gov. Should you have questions on access to the Private PAIR system, contact the Electronic Business Center (EBC) at 866-217-9197 (toll-free). If you would like assistance from a USPTO Customer Service Representative or access to the automated information system, call 800-786-9199 (IN USA OR CANADA) or 571-272-1000.

/Angela M. Bertagna/
Primary Examiner, Art Unit 1637

Notice of References Cited	Application/Control No. 14/067,620	Applicant(s)/Patent Under Reexamination MAPLES ET AL.	
	Examiner Angela M. Bertagna	Art Unit 1637	Page 1 of 1

U.S. PATENT DOCUMENTS

*		Document Number Country Code-Number-Kind Code	Date MM-YYYY	Name	Classification
*	A	US-2009/0092967 A1	04-2009	Yao et al.	435/6
	B	US-			
	C	US-			
	D	US-			
	E	US-			
	F	US-			
	G	US-			
	H	US-			
	I	US-			
	J	US-			
	K	US-			
	L	US-			
	M	US-			

FOREIGN PATENT DOCUMENTS

*		Document Number Country Code-Number-Kind Code	Date MM-YYYY	Country	Name	Classification
	N					
	O					
	P					
	Q					
	R					
	S					
	T					

NON-PATENT DOCUMENTS

*		Include as applicable: Author, Title Date, Publisher, Edition or Volume, Pertinent Pages)
	U	
	V	
	W	
	X	

*A copy of this reference is not being furnished with this Office action. (See MPEP § 707.05(a).)
Dates in MM-YYYY format are publication dates. Classifications may be US or foreign.

EAST Search History

EAST Search History (Prior Art)

Ref #	Hits	Search Query	DBs	Default Operator	Plurals	Time Stamp
S48	262	(sda or (strand adj1 displacement adj1 amplification)) same (betaine or dmso or formamide)	US-PGPUB; USPAT; FPRS; EPO; JPO; DERWENT	OR	OFF	2015/02/18 12:54
S49	45	(sda or (strand adj1 displacement adj1 amplification)) same (betaine or dmso or formamide) same denat$	US-PGPUB; USPAT; FPRS; EPO; JPO; DERWENT	OR	OFF	2015/02/18 12:54
S50	54	(sda or (strand adj1 displacement adj1 amplification)) same (("without" or "no") near2 denat$)	US-PGPUB; USPAT; FPRS; EPO; JPO; DERWENT	OR	OFF	2015/02/18 13:00
S51	2	"6033881".pn. "6087133".pn. us-20030165939-$.did.	USPAT	OR	OFF	2015/02/19 10:30
S52	1	"6033881".pn. "6087133".pn. us-20030165939-$.did.	US-PGPUB	OR	OFF	2015/02/19 10:44
S53	5	("7972820" | "6632611" | "7270981" | "7399590" | "6656680").PN.	USPAT	OR	OFF	2015/02/19 18:09
S54	1	"0678582".pn. us-2010255546-$.did.	US-PGPUB; USPAT	OR	OFF	2015/02/19 18:40
S55	3	"0678582".pn. us-2010255546-$.did.	US-PGPUB; USPAT; FPRS; EPO; JPO; DERWENT	OR	OFF	2015/02/19 18:40
S56	5	"0678582".pn. us-20100255546-$.did.	US-PGPUB; USPAT; FPRS; EPO; JPO; DERWENT	OR	OFF	2015/02/19 18:41
S57	22	("20100255546" | "20110171649" | "20120015358" | "20120244535" | "4943522" | "5270184" | "5422252" | "5455166" | "5470723" | "5561944" | "5624825" | "5712124" | "5736365" | "5824796" | "5912340" | "6063604" | "6127121" | "6660845" | "6683173" | "7045610" | "7282328" | "7488578" | "7751982" | "7799554" | "8202972" | "RE39885").PN.	USPAT	OR	OFF	2015/02/19 18:46

S58	4	("20100255546" | "20110171649" | "20120015358" | "20120244535" | "4943522" | "5270184" | "5422252" | "5455166" | "5470723" | "5561944" | "5624825" | "5712124" | "5736365" | "5824796" | "5912340" | "6063604" | "6127121" | "6660845" | "6683173" | "7045610" | "7282328" | "7488578" | "7751982" | "7799554" | "8202972" | "RE39885").PN.	US-PGPUB	OR	OFF	2015/02/19 18:46
S59	9	(ionian).as. and (nicking or nick or nicked or nickase)	US-PGPUB; USPAT; FPRS; EPO; JPO; DERWENT	OR	OFF	2015/02/19 18:49
S60	15	(brian near2 maples).in.	US-PGPUB; USPAT; FPRS; EPO; JPO; DERWENT	OR	OFF	2015/02/19 18:49
S61	24	(rebecca near2 holmberg).in.	US-PGPUB; USPAT; FPRS; EPO; JPO; DERWENT	OR	OFF	2015/02/19 18:50
S62	9	S61 not S60	US-PGPUB; USPAT; FPRS; EPO; JPO; DERWENT	OR	OFF	2015/02/19 18:50
S63	2273	(andrew near2 miller).in.	US-PGPUB; USPAT; FPRS; EPO; JPO; DERWENT	OR	OFF	2015/02/19 18:50
S64	4	(andrew near2 miller).in. and (nicking or nick or nicked or nicks or nickase).clm.	US-PGPUB; USPAT; FPRS; EPO; JPO; DERWENT	OR	OFF	2015/02/19 18:50
S65	17	(Provins near2 Jarrod)".in. "	US-PGPUB; USPAT; FPRS; EPO; JPO; DERWENT	OR	OFF	2015/02/19 18:51
S66	22	S65 not S64 S61 not S60	US-PGPUB; USPAT; FPRS; EPO; JPO; DERWENT	OR	OFF	2015/02/19 18:51
S67	2	S65 not S64 not S61 not S60	US-PGPUB; USPAT;	OR	OFF	2015/02/19 18:51

			FPRS; EPO; JPO; DERWENT			
S68	628	(richard near2 roth).in.	US-PGPUB; USPAT; FPRS; EPO; JPO; DERWENT	OR	OFF	2015/02/19 18:51
S69	5	(richard near2 roth).in. and (nicking or nick or nicked or nicks or nickase).clm.	US-PGPUB; USPAT; FPRS; EPO; JPO; DERWENT	OR	OFF	2015/02/19 18:52
S70	0	(rich near2 roth).in. and (nicking or nick or nicked or nicks or nickase).clm.	US-PGPUB; USPAT; FPRS; EPO; JPO; DERWENT	OR	OFF	2015/02/19 18:52
S71	15	(jeffrey near2 mandell).in.	US-PGPUB; USPAT; FPRS; EPO; JPO; DERWENT	OR	OFF	2015/02/19 18:52

EAST Search History (Interference)

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2/19/2015 6:54:33 PM
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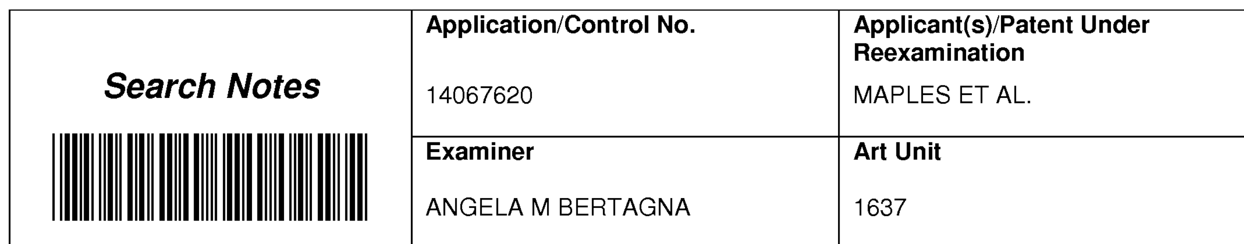

Search Notes	Application/Control No. 14067620	Applicant(s)/Patent Under Reexamination MAPLES ET AL.
	Examiner ANGELA M BERTAGNA	Art Unit 1637

CPC- SEARCHED		
Symbol	Date	Examiner

CPC COMBINATION SETS - SEARCHED		
Symbol	Date	Examiner

US CLASSIFICATION SEARCHED			
Class	Subclass	Date	Examiner

SEARCH NOTES		
Search Notes	Date	Examiner
searched all inventors by name	10/10/2014	amb
EAST search history attached	10/10/2014; 10/14/2014	amb
Google Scholar (search terms included "nicking", "isothermal", and "RNA")	10/10/2014; 10/14/2014	amb
reviewed related cases - 14067623 & 11778018	10/10/2014	amb
updated search	2/18/15; 2/19/15	amb

INTERFERENCE SEARCH			
US Class/ CPC Symbol	US Subclass / CPC Group	Date	Examiner

Substitute Disclosure Form U.S. Department of Commerce Patent and Trademark Office	Attorney Docket No. 30171-0025002	Application No. 14/067,620
Information Disclosure Statement by Applicant (Use several sheets if necessary)	Applicant Ionian Technologies Inc.	
(37 CFR §1.98(b))	Filing Date October 30, 2013	Group Art Unit 1637

U.S. Patent Documents

Examiner Initial	Desig. ID	Document Number	Publication Date	Patentee	Class	Subclass	Filing Date If Appropriate
/A.B./	1	7,972,820	7/5/2011	Mayer			

Foreign Patent Documents or Published Foreign Patent Applications

Examiner Initial	Desig. ID	Document Number	Publication Date	Country or Patent Office	Class	Subclass	Translation Yes	Translation No
	2							

Other Documents (include Author, Title, Date, and Place of Publication)

Examiner Initial	Desig. ID	Document
/A.B./	3	Notice of Allowance in corresponding Japanese Application No. 2010-517111, dated January 15, 2015, pages 1-3
/A.B./	4	Response to Examination Report in corresponding Australian Application No. 2008276118, dated January 23, 2015, pages 1-5
/A.B./	5	Response to Office Action dated July 31, 2014, for corresponding Canadian Application No. 2693805, dated January 30, 2015, pages 1-62
	6	

Examiner Signature /Angela Bertagna/	Date Considered 02/19/2015
EXAMINER: Initials citation considered. Draw line through citation if not in conformance and not considered. Include copy of this form with next communication to applicant.	

Substitute Disclosure Form

Substitute Disclosure Form U.S. Department of Commerce Patent and Trademark Office	Attorney Docket No. 30171-0025002	Application No. 14/067,620
Information Disclosure Statement by Applicant (Use several sheets if necessary)	Applicant Ionian Technologies Inc.	
(37 CFR §1.98(b))	Filing Date October 30, 2013	Group Art Unit 1637

U.S. Patent Documents

Examiner Initial	Desig. ID	Document Number	Publication Date	Patentee	Class	Subclass	Filing Date If Appropriate
	1						

Foreign Patent Documents or Published Foreign Patent Applications

Examiner Initial	Desig. ID	Document Number	Publication Date	Country or Patent Office	Class	Subclass	Translation	
							Yes	No
	2							

Other Documents (include Author, Title, Date, and Place of Publication)

Examiner Initial	Desig. ID	Document
/A.B./	3	Walker et al., "Isothermal *in vitro* amplification of DNA by a restriction enzyme/DNA polymerase system," PNAS, 89:392-396 (1992)
	4	

Examiner Signature /Angela Bertagna/	Date Considered 02/19/2015
EXAMINER: Initials citation considered. Draw line through citation if not in conformance and not considered. Include copy of this form with next communication to applicant.	

Substitute Disclosure Form

Substitute Disclosure Form U.S. Department of Commerce Patent and Trademark Office	Attorney Docket No. 30171-0025002	Application No. 14/067,620
Information Disclosure Statement by Applicant (Use several sheets if necessary)	Applicant Ionian Technologies Inc.	
(37 CFR §1.98(b))	Filing Date October 30, 2013	Group Art Unit 1637

U.S. Patent Documents

Examiner Initial	Desig. ID	Document Number	Publication Date	Patentee	Class	Subclass	Filing Date If Appropriate
/A.B./	1	7,270,981	9/18/2007	Armes et al.			
/A.B./	2	7,399,590	7/15/2008	Piepenburg et al.			
	3						

Foreign Patent Documents or Published Foreign Patent Applications

Examiner Initial	Desig. ID	Document Number	Publication Date	Country or Patent Office	Class	Subclass	Translation Yes	Translation No
	4							

Other Documents (include Author, Title, Date, and Place of Publication)

Examiner Initial	Desig. ID	Document
/A.B./	5	Response of Patentee to Opposition in corresponding EP Application No. 08781827.4 (EP 2 181 196), dated January 7, 2015, pages 1-36.

Examiner Signature /Angela Bertagna/	Date Considered 02/19/2015
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Substitute Disclosure Form

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

First Named Inventor	:	Brian K. Maples	Art Unit	:	1637
Serial No.	:	14/067,620	Examiner	:	Olayinka A. Oyeyemi
Filed	:	October 30, 2013	Conf. No.	:	4288
Title	:	NICKING AND EXTENSION AMPLIFICATION REACTION FOR THE EXPONENTIAL AMPLIFICATION OF NUCLEIC ACIDS			

MAIL STOP AMENDMENT
Commissioner for Patents
P.O. Box 1450
Alexandria, VA 22313-1450

INFORMATION DISCLOSURE STATEMENT

Please consider the references listed on the enclosed PTO-SB-08 or Disclosure Form. Foreign patent documents and non-patent literature are enclosed; cited U.S. patents and patent application publications will be provided on request. A copy of a communication from a foreign patent office in a counterpart application is also enclosed.

This statement is being filed after a first action on the merits, but before receipt of a final action or a notice of allowance. A late submission fee in the amount of $180, specified by 37 CFR §1.17(p), is being paid with this statement.

Apply any necessary charges or credits to deposit account 06-1050, referencing the above attorney docket number.

Respectfully submitted,

Date: February 2, 2015 ______________

/Ian J.S. Lodovice, Reg. No. 59,749/__________
Ian J. Lodovice
Reg. No. 59,749

Customer Number 26161
Fish & Richardson P.C.
Telephone: (617) 956-5972
Facsimile: (877) 769-7945

23350861.doc

Substitute Disclosure Form	U.S. Department of Commerce Patent and Trademark Office	Attorney Docket No. 30171-0025002	Application No. 14/067,620
Information Disclosure Statement by Applicant (Use several sheets if necessary)		Applicant Ionian Technologies Inc.	
(37 CFR §1.98(b))		Filing Date October 30, 2013	Group Art Unit 1637

U.S. Patent Documents

Examiner Initial	Desig. ID	Document Number	Publication Date	Patentee	Class	Subclass	Filing Date If Appropriate
	1	7,972,820	7/5/2011	Mayer			

Foreign Patent Documents or Published Foreign Patent Applications

Examiner Initial	Desig. ID	Document Number	Publication Date	Country or Patent Office	Class	Subclass	Translation	
							Yes	No
	2							

Other Documents (include Author, Title, Date, and Place of Publication)

Examiner Initial	Desig. ID	Document
	3	Notice of Allowance in corresponding Japanese Application No. 2010-517111, dated January 15, 2015, pages 1-3
	4	Response to Examination Report in corresponding Australian Application No. 2008276118, dated January 23, 2015, pages 1-5
	5	Response to Office Action dated July 31, 2014, for corresponding Canadian Application No. 2693805, dated January 30, 2015, pages 1-62
	6	

Examiner Signature	Date Considered
EXAMINER: Initials citation considered. Draw line through citation if not in conformance and not considered. Include copy of this form with next communication to applicant.	

Substitute Disclosure Form

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

First Named Inventor	:	Brian K. Maples	Art Unit	:	1637
Serial No.	:	14/067,620	Examiner	:	Olayinka A. Oyeyemi
Filed	:	October 30, 2013	Conf. No.	:	4288
Title	:	NICKING AND EXTENSION AMPLIFICATION REACTION FOR THE EXPONENTIAL AMPLIFICATION OF NUCLEIC ACIDS			

MAIL STOP AMENDMENT
Commissioner for Patents
P.O. Box 1450
Alexandria, VA 22313-1450

INFORMATION DISCLOSURE STATEMENT

Please consider the references listed on the enclosed PTO-SB-08 or Disclosure Form. Foreign patent documents and non-patent literature are enclosed; cited U.S. patents and patent application publications will be provided on request. A copy of a communication from a foreign patent office in a counterpart application is also enclosed.

This statement is being filed after a first action on the merits, but before receipt of a final action or a notice of allowance. A late submission fee in the amount of $180, specified by 37 CFR §1.17(p), is being paid with this statement.

Apply any necessary charges or credits to deposit account 06-1050, referencing the above attorney docket number.

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Information Disclosure Statement by Applicant (Use several sheets if necessary) (37 CFR §1.98(b))	Applicant Ionian Technologies Inc.	
	Filing Date October 30, 2013	Group Art Unit 1637

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	1	7,972,820	7/5/2011	Mayer			

Foreign Patent Documents or Published Foreign Patent Applications

Examiner Initial	Desig. ID	Document Number	Publication Date	Country or Patent Office	Class	Subclass	Translation Yes	Translation No
	2							

Other Documents (include Author, Title, Date, and Place of Publication)

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	6	

Examiner Signature	Date Considered
EXAMINER: Initials citation considered. Draw line through citation if not in conformance and not considered. Include copy of this form with next communication to applicant.	

Substitute Disclosure Form

Electronic Patent Application Fee Transmittal

Application Number:	14067620
Filing Date:	30-Oct-2013
Title of Invention:	Nicking and Extension Amplification Reaction for the Exponential Amplification of Nucleic Acids
First Named Inventor/Applicant Name:	Brian K. Maples
Filer:	Ian J.S. Lodovice/Mary Florczak
Attorney Docket Number:	30171-0025002 / ITI-001

Filed as Large Entity

Filing Fees for **Utility under 35 USC 111(a)**

Description	Fee Code	Quantity	Amount	Sub-Total in USD($)
Basic Filing:				
Pages:				
Claims:				
Miscellaneous-Filing:				
Petition:				
Patent-Appeals-and-Interference:				
Post-Allowance-and-Post-Issuance:				
Extension-of-Time:				

Description	Fee Code	Quantity	Amount	Sub-Total in USD($)
Miscellaneous:				
Submission- Information Disclosure Stmt	1806	1	180	180
	Total in USD ($)			**180**

Electronic Acknowledgement Receipt

EFS ID:	21376892
Application Number:	14067620
International Application Number:	
Confirmation Number:	4288
Title of Invention:	Nicking and Extension Amplification Reaction for the Exponential Amplification of Nucleic Acids
First Named Inventor/Applicant Name:	Brian K. Maples
Customer Number:	26161
Filer:	Ian J.S. Lodovice/Mary Florczak
Filer Authorized By:	Ian J.S. Lodovice
Attorney Docket Number:	30171-0025002 / ITI-001
Receipt Date:	02-FEB-2015
Filing Date:	30-OCT-2013
Time Stamp:	18:44:11
Application Type:	Utility under 35 USC 111(a)

Payment information:

Submitted with Payment	yes
Payment Type	Deposit Account
Payment was successfully received in RAM	$180
RAM confirmation Number	5057
Deposit Account	061050
Authorized User	

The Director of the USPTO is hereby authorized to charge indicated fees and credit any overpayment as follows:

File Listing:

Document Number	Document Description	File Name	File Size(Bytes)/ Message Digest	Multi Part /.zip	Pages (if appl.)
1	Information Disclosure Statement (IDS) Form (SB08)	301710025IDS.pdf	158122 885b559c90a1d6fe55acaf179f1c0a08c7e54a5d	no	2
Warnings:					
Information:					
This is not an USPTO supplied IDS fillable form					
2	Non Patent Literature	AUResponse.pdf	167102 59fd470ede67f12a3b6e19dcbe77f9beb6f8857e	no	5
Warnings:					
Information:					
3	Non Patent Literature	CAResponse.pdf	3508203 14ca0a19ed3f4356068238620aee980409355719	no	62
Warnings:					
Information:					
4	Non Patent Literature	JPNOA.pdf	41051 4f62d700f8cdba2b058c1990f465c661c613632a	no	3
Warnings:					
Information:					
5	Fee Worksheet (SB06)	fee-info.pdf	30661 03bcf549b52dcb9901dbef7b95a2f573baa6d116	no	2
Warnings:					
Information:					
		Total Files Size (in bytes):	3905139		

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New Applications Under 35 U.S.C. 111

If a new application is being filed and the application includes the necessary components for a filing date (see 37 CFR 1.53(b)-(d) and MPEP 506), a Filing Receipt (37 CFR 1.54) will be issued in due course and the date shown on this Acknowledgement Receipt will establish the filing date of the application.

National Stage of an International Application under 35 U.S.C. 371

If a timely submission to enter the national stage of an international application is compliant with the conditions of 35 U.S.C. 371 and other applicable requirements a Form PCT/DO/EO/903 indicating acceptance of the application as a national stage submission under 35 U.S.C. 371 will be issued in addition to the Filing Receipt, in due course.

New International Application Filed with the USPTO as a Receiving Office

If a new international application is being filed and the international application includes the necessary components for an international filing date (see PCT Article 11 and MPEP 1810), a Notification of the International Application Number and of the International Filing Date (Form PCT/RO/105) will be issued in due course, subject to prescriptions concerning national security, and the date shown on this Acknowledgement Receipt will establish the international filing date of the application.

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

First Named Inventor	:	Brian K. Maples	Art Unit	:	1637
Serial No.	:	14/067,620	Examiner	:	Angela Marie Bertagna
Filed	:	October 30, 2013	Conf. No.	:	4288
Title	:	NICKING AND EXTENSION AMPLIFICATION REACTION FOR THE EXPONENTIAL AMPLIFICATION OF NUCLEIC ACIDS			

Mail Stop Amendment
Commissioner for Patents
P.O. Box 1450
Alexandria, VA 22313-1450

REPLY TO ACTION OF OCTOBER 20, 2014

Please consider the following reply.

Amendments to the Claims:

This listing of claims replaces all prior versions and listings of claims in the application:

Listing of Claims:

1. - 66. (Canceled)

67. (Currently Amended) A method of amplifying a target polynucleotide sequence of a target nucleic acid present in a sample obtained from an animal, the method comprising:

(a) preparing, without first subjecting the target nucleic acid to a denaturation step associated with amplification of the target polynucleotide sequence, a mixture comprising:

(i) [[a]] the target nucleic acid ~~present in a sample obtained from an animal, the target nucleic acid having a~~ comprising the target polynucleotide sequence,

(ii) a polymerase,

(iii) a nicking enzyme,

(iv) a first oligonucleotide comprising a nicking site and a nicking enzyme binding site, and

(v) a second oligonucleotide comprising a nicking site and a nicking enzyme binding site, and

(b) subjecting the mixture to essentially isothermal conditions to amplify the target polynucleotide sequence.

68. (Previously Presented) The method of claim 67, wherein the target polynucleotide sequence is amplified from steps comprising:

(a) forming a first duplex comprising the target polynucleotide sequence and the first oligonucleotide;

(b) extending, using the polymerase, the first oligonucleotide along the target polynucleotide sequence to form an extended first oligonucleotide comprising a sequence complementary to the second oligonucleotide;

(c) forming a second duplex comprising the second oligonucleotide and the extended first oligonucleotide;

(d) extending, using the polymerase, the second oligonucleotide along the extended first oligonucleotide to form a third duplex comprising an extended second oligonucleotide comprising a sequence complementary to the first oligonucleotide and a first double-stranded nicking enzyme binding site;

(e) nicking, with the nicking enzyme, the first nicking site on the third duplex to produce a fourth duplex comprising the extended second oligonucleotide and a fragment of the extended first oligonucleotide; and

(f) extending, using the polymerase, the fragment of the extended first oligonucleotide along the extended second oligonucleotide of the fourth duplex to produce a double-stranded nucleic acid product and a second double-stranded nicking enzyme binding site.

69. (Previously Presented) The method of claim 68, wherein the double-stranded nucleic acid product comprises:

i) a first strand and a second strand, wherein the first strand comprises a first polynucleotide sequence corresponding to the target polynucleotide sequence and the second strand comprises a second polynucleotide sequence complementary to the target polynucleotide sequence, and

ii) first and second double-stranded nicking sites spaced apart by the target polynucleotide sequence.

70. (Previously Presented) The method of claim 68, further comprising the steps of:

a) nicking, using the nicking enzyme, the first nicking site of the double-stranded nucleic acid product to produce a fifth duplex comprising a first polynucleotide sequence corresponding to the target polynucleotide sequence and a fragment of the first oligonucleotide, and nicking, using the nicking enzyme, the second nicking site of the double-stranded nucleic acid product to

produce a sixth duplex comprising a second polynucleotide sequence complementary to the target polynucleotide sequence and a fragment of the second oligonucleotide;

b) extending, using the polymerase, the fragment of the first oligonucleotide along the first polynucleotide sequence of the fifth duplex to produce a first double stranded product comprising a copy of the nicking site and a copy of the first polynucleotide sequence and extending, using the polymerase, the fragment of the second oligonucleotide along the second polynucleotide sequence of the sixth duplex to produce a second double stranded product comprising a copy of the nicking site and a copy of the second polynucleotide sequence; and

c) nicking, using the nicking enzyme, the copy of the nicking site of the first double stranded product to release a copy of the first polynucleotide sequence and nicking, using the nicking enzyme, the copy of the nicking site of the second double stranded product to release a copy of the second polynucleotide sequence.

71. (Previously Presented) The method of claim 67, wherein the animal is a human.

72. (Previously Presented) The method of claim 67, wherein the target nucleic acid is obtained from an animal pathogen.

73. (Previously Presented) The method of claim 72, wherein the animal pathogen is a single-stranded DNA virus, double-stranded DNA virus, or single-stranded RNA virus.

74. (Withdrawn) The method of claim 72, wherein the animal pathogen is a bacterium.

75. (Withdrawn) The method of claim 72, wherein the animal pathogen contains spores and the target polynucleotide is amplified from the spores without the need for lysis of the spores.

76. (Currently Amended) The method of claim 67, wherein the sample obtained from an animal is obtained from the blood, bone marrow, mucus, lymph, hard tissues ~~(e.g. liver, spleen, kidney, lung or ovary)~~, biopsies, sputum, saliva, tears, faeces or urine of the animal.

77. (Previously Presented) The method of claim 76, wherein the sample obtained from an animal is obtained from the mucus, sputum, or saliva of the animal.

78. (Withdrawn) The method of claim 67, wherein the target nucleic acid is double-stranded DNA.

79. (Withdrawn) The method of claim 67, wherein the target nucleic acid is single-stranded DNA.

80. (Previously Presented) The method of claim 67, wherein the target nucleic acid is RNA.

81. (Withdrawn) The method of claim 67, wherein the target nucleic acid is selected from the group consisting of genomic DNA, plasmid DNA, viral DNA, mitochondrial DNA, cDNA, synthetic double-stranded DNA and synthetic single-stranded DNA.

82. (Withdrawn) The method of claim 81, wherein the target nucleic acid is genomic DNA.

83. (Previously Presented) The method of claim 67, wherein the target nucleic acid is viral DNA or viral RNA.

84. (Canceled)

85. (Previously Presented) The method of claim 67, wherein the nicking enzyme is Nt.BstNBI.

86. (Previously Presented) The method of claim 67, wherein the nicking enzyme does not nick within the target polynucleotide sequence.

87. (Currently Amended) The method of claim 67, ~~which~~ wherein amplification of the target polynucleotide sequence is performed without the use of temperature cycling.

88. (Currently Amended) The method of claim 67, ~~which~~ wherein amplification of the target polynucleotide sequence is performed at about 55°C-59°C.

89. (Currently Amended) The method of claim 67, ~~which~~ wherein amplification of the target polynucleotide sequence is performed at a constant temperature for about 1 to 20 minutes.

90. (Previously Presented) The method of claim 68, which is performed at a temperature higher than the melting temperature of the first oligonucleotide/target polynucleotide sequence complex.

91. (Previously Presented) The method of claim 67, further comprising detecting amplification product.

92. (Currently Amended) The method of claim 91, wherein the amplification product is detected by a detection method selected from the group consisting of gel electrophoresis, mass spectrometry, ~~SYBR I~~ fluorescence, ~~SYBR II fluorescence, SYBR Gold, Pico Green, TOTO-3,~~ intercalating dye detection, fluorescence resonance energy transfer (FRET), molecular beacon detection, surface capture, capillary electrophoresis, incorporation of labeled nucleotides to allow detection by capture, fluorescence polarization, and lateral flow capture, or a combination thereof.

93. (Canceled)

94. (Canceled)

95. (Withdrawn, Currently Amended) A method of amplifying a target polynucleotide sequence of genomic DNA present in a sample obtained from an animal, the method comprising:

(a) preparing, without first subjecting the genomic DNA to a denaturation step associated with amplification of the target polynucleotide sequence, a mixture comprising:

(i) the genomic DNA ~~present in a sample obtained from an animal, the genomic DNA having a~~ comprising the target polynucleotide sequence,

(ii) a polymerase,

(iii) a nicking enzyme,

(iv) a first oligonucleotide comprising a nicking site and a nicking enzyme binding site, and

(v) a second oligonucleotide comprising a nicking site and a nicking enzyme binding site, and

b) subjecting the mixture to essentially isothermal conditions to amplify the target polynucleotide sequence~~; which method is performed without an initial heat denaturation step~~.

REMARKS

Upon entry of the above amendment, claims 67-83, 85-92 and 95 will be pending. Claims 1-66 were previously canceled, and claims 84 and 93-94 are newly canceled. Claims 74-75, 78-79, 81-82 and 95 were previously withdrawn, so are not currently under examination. Claims 67, 76, 87-89 and 92 (and withdrawn claim 95) have been amended. Most of the amendments are simply to clarify scope and are supported throughout the specification.

For example, the amendment to claims 67 and 95 find support at page 4, lines 16-29; Figures 1A-D; and throughout the examples. Claims 76 was amended for clarity, and claims 87-89 were amended for consistency with amended claim 67.

No new matter has been introduced by these amendments. Reconsideration and allowance of the claims are respectfully requested in view of the above amendments and the following remarks.

The present application does contain nonelected and withdrawn claims, claims 74-75, 78-79, 81-82 and 95. Applicant believes that these claims will be eligible for rejoinder upon allowance of a generic claim. Thus, they are being maintained in the present application.

Interview Summary

Applicant thanks Examiner Bertagna for the courtesy of an in-person interview with Applicant's representatives Ian Lodovice, Rich Roth, Julius Fister, and Belinda Lew on December 8, 2014. During the interview, the participants discussed the rejections under 35 U.S.C. §§ 102(b) and 103(a) and amendments for placing claims in conditions for allowance. The amendments to claims presented above are substantially as discussed by the participants during the interview. Applicant acknowledges and thanks Examiner Bertagna for the Applicant-Initiated Interview Summary dated December 12, 2014.

Drawings

The replacement drawings filed on December 18, 2013 are objected because Figures 17 and 18 are not labeled as "Replacement Drawings." Submitted herewith are replacement drawings, including Figures 17 and 18, in compliance with 37 CFR § 1.84.

Specification

The specification is objected as allegedly not providing antecedent basis for the subject matter of claim 93. Without conceding the appropriateness of the objection and solely in the interest of advancing prosecution, claim 93 is being canceled. Withdrawal of the objection is requested.

Claim Rejections Under 35 U.S.C. § 112, second paragraph

Claims 76, 77, 84 and 87-94 stand rejected under 35 U.S.C. § 112, second paragraph, as allegedly being indefinite for failing to particularly point out and distinctly claim the subject matter which the applicant regards as the invention. Applicants submit that the claims as amended meet the requirements of 35 U.S.C. § 112, second paragraph. Reconsideration and withdrawal of the rejection of under 35 U.S.C. 112, second paragraph is requested.

Claim Rejections Under 35 U.S.C. §§ 102(b) and 103(a)

The claims presented herein require the steps of (a) preparing a mixture comprising a target nucleic acid having a target polynucleotide sequence and amplification reagents, and (b) subjecting the mixture to essentially isothermal conditions to amplify the target polynucleotide sequence. The claims further require that prior to step (a), the target nucleic acid is not subjected to a step associated with amplification that is sufficient to denature it.

As discussed with the Examiner during the interview, the invention, as set forth in the claims herein, permits one to amplify a target polynucleotide sequence of a target nucleic acid, without first subjecting the target nucleic acid to a step associated with amplification that is sufficient to denature the target nucleic acid. One may, for example, take a sample containing a target sequence and, without prior denaturation of the target nucleic acid, combine it with all

reagents needed for amplification and proceed through amplification under essentially isothermal conditions.

In contrast to the present invention as claimed, the cited references expressly teach a denaturation step in connection with amplification, for example a heat denaturation step or PCR-suitable temperature thermocycling, prior to the step (a) of preparing the mixture. There is simply no motivation or other reason to modify the cited references to omit such a denaturation step prior to essentially isothermal amplification and furthermore, there would have not have been a reasonable expectation of success in modifying the prior art to omit such denaturation.[1]

As further evidence of the novel and inventive features of the claimed invention, the Applicant has now not only received 510(k) approval from the United States Food and Drug Administration (FDA) for marketing a product in the United States that incorporates the technology, Applicant is also the first to be granted a Clinical Laboratory Improvement Amendments (CLIA) waiver from the FDA for a nucleic acid-based test.

Turning to the specific references cited by the Examiner:

1. Claims 67, 71-73, 76, 80, 83, 85, and 86 stand rejected under 102(b) as allegedly anticipated by Van Ness et al. (WO 2003/008642)("Van Ness").

Claim 67 requires the step of (a) preparing a mixture comprising a target nucleic acid and a nicking enzyme, without first subjecting the target nucleic acid to a denaturation step associated with amplification of the target polynucleotide sequence. After preparation of the mixture, the mixture is subjected to essentially isothermal conditions to amplify the target polynucleotide sequence.

Van Ness fails to disclose the method of claim 67. For example, provided below is a reproduction of Figure 2 of Van Ness.

[1] Applicant submits that the present claims are patentable even if the prior art were so modified.

<u>Figure 2, Van Ness</u>

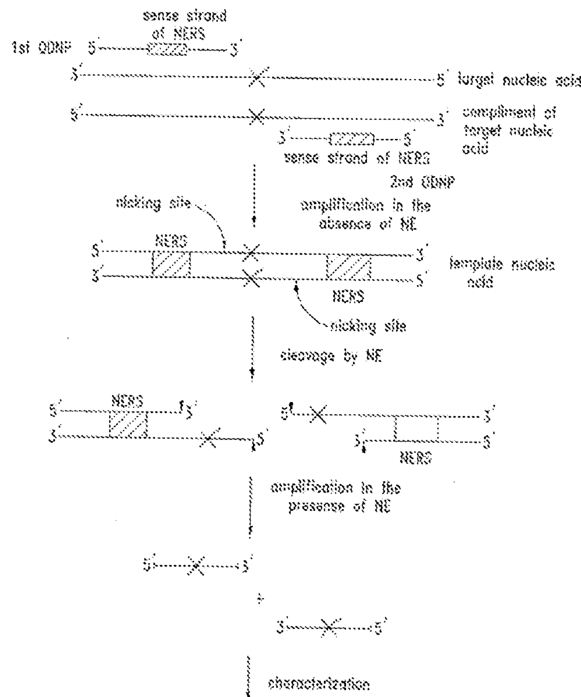

Fig. 2

As shown in Figure 2 of Van Ness, nicking enzymes are combined with a so-called "template nucleic acid"[2] that has been prepared by "amplification in the absence of NE", i.e., amplification in the absence of nicking enzymes.

The omission of nicking enzymes from this first, "prior" amplification step shown in Figure 2 is hardly surprising since Van Ness describes only PCR- or LCR-based amplification for the production of the "template nucleic acid." For example, see Van Ness page 91, line 8 to page 93, line 28.

Both PCR and LCR require temperature thermocycling that is sufficient to denature the target nucleic acid. Therefore, in contrast to the present claims, Van Ness <u>requires a step that is</u>

[2] Applicant does not concede that the "template nucleic acid" in Van Ness is the same as the "templates" in independent claim 67.

sufficient to denature the target nucleic acid, prior to combining the target nucleic acid with nicking enzymes.

Accordingly, Van Ness does not perform the present claimed step (a) of preparing a mixture comprising a target nucleic acid and a nicking enzyme, because that step (a), as required by claims 67, must be performed "without first subjecting the target nucleic acid to a denaturation step associated with amplification of the target polynucleotide sequence". Van Ness does not add nicking enzymes until **after** a denaturation step associated with "amplification in the absence of NE."

For at least these reasons, Van Ness does not anticipate independent claim 67. Because independent claim 67 is patentable over Van Ness, dependent claims 71-73, 76, 80, 83, 85, and 86 are also patentable over Van Ness. For at least these reasons, Applicant requests reconsideration and withdrawal of this rejection.

2. Claims 67-73, 76, 77, 80, and 83-94 stand rejected under 103(a) as allegedly unpatentable over Wick et al. (US 6,063,604)("Wick") in view of Kong et al. (US 6,191,267)("Kong").

Claim 67 requires the step of (a) preparing a mixture comprising a target nucleic acid and a nicking enzyme, without first subjecting the target nucleic acid to a denaturation step associated with amplification of the target polynucleotide sequence. After preparation of the mixture, the mixture is subjected to essentially isothermal conditions to amplify the target polynucleotide sequence.

Wick fails to disclose the method of claim 67. In all Wick's Examples, a denaturation step is required to prepare the target nucleic acid for subsequent amplification. See, for example, Wick, col. 29, lines 1-2; Wick, col. 30, lines 25-26; Wick, col. 31, lines 5-6; and Wick, col. 32, lines 19-20.

Kong fails to remedy the deficiencies of Wick. Kong also requires first subjecting the target DNA to a denaturation step, similar to Wick and in contrast to present claim 67. See, for example, Kong at col. 14, line 29.

Moreover, even if the combination of Wick and Kong somehow discloses all the features of present claim 67 (which Applicant submits is not the case), there is no prima facie case of

obviousness because there is no teaching, suggestion, or motivation to combine Wick and Kong in the manner indicated by the Examiner. Kong's disclosure of a nucleic acid amplification process is limited to a single Example involving a double-stranded DNA target, in contrast to Wick, in which all the Examples are of single-stranded RNA targets. Moreover, Kong requires at least four primers (see Kong, Example 4), in contrast to Wick, which teaches no more than two primers in all the Examples. Even if there was motivation to combine Wick and Kong (and Applicant submits there is not), there is no reasonable expectation of success that the disparate teachings of the two references could even be successfully combined to arrive at the claimed invention.

For at least these reasons, independent claim 67 is patentable in view of the combination of Wick and Kong. Because independent claim 67 is patentable over Wick and Kong, dependent claims 68-73, 76, 77, 80, and 83-94 are also patentable over Wick and Kong. For at least these reasons, Applicant requests reconsideration and withdrawal of this rejection.

Double Patenting

Claims 67-73, 76, 77, 80, and 83-94 were provisionally rejected on the ground of nonstatutory double patenting as allegedly being unpatentable over claims 67-69, 74-76, 79, 80, 83, and 85-96 of copending Application No. 14/067,623.

Claims 67-73, 76, 77, 80, and 83-94 were provisionally rejected on the ground of nonstatutory double patenting as allegedly being unpatentable over claims 1-9, 12, 14-17, 19-42, and 44- 47 of copending Application No. 12/173,020.

Claims 67-73, 76, 77, 80, 83, 84, 86-88, and 90-94 were provisionally rejected on the ground of nonstatutory double patenting as allegedly being unpatentable over claims 125-130 of copending Application No. 11/778,018 in view of Wick.

Claims 85 and 89 were also provisionally rejected on the ground of nonstatutory double patenting as allegedly being unpatentable over claims 125-130 of copending Application No. 11/778,018 in view of Wick and further in view of Kong.

Applicant does not concede that the rejections above are appropriate. Further, as the applications cited above are all currently pending, Applicant requests that the rejections be held in abeyance pending the identification of allowable subject matter.

CONCLUSION

In light of the arguments made herein, Applicant submits that the pending claims are patentable and request early and favorable action thereon. If the Examiner feels it would further prosecution of the present case, he is invited to telephone the undersigned at 617-956-5972.

Applicant does not concede any positions of the Office that are not expressly addressed above, nor does Applicant concede that there are not other good reasons for patentability of the presented claims or other claims.

Please apply any necessary charges or credits to Deposit Account 06 1050, referencing the attorney docket number 30171-0025002.

Respectfully submitted,

Date: January 9, 2015________________

/Ian J.S. Lodovice, Reg. No. 59,749/__________
Ian J. Lodovice
Reg. No. 59,749

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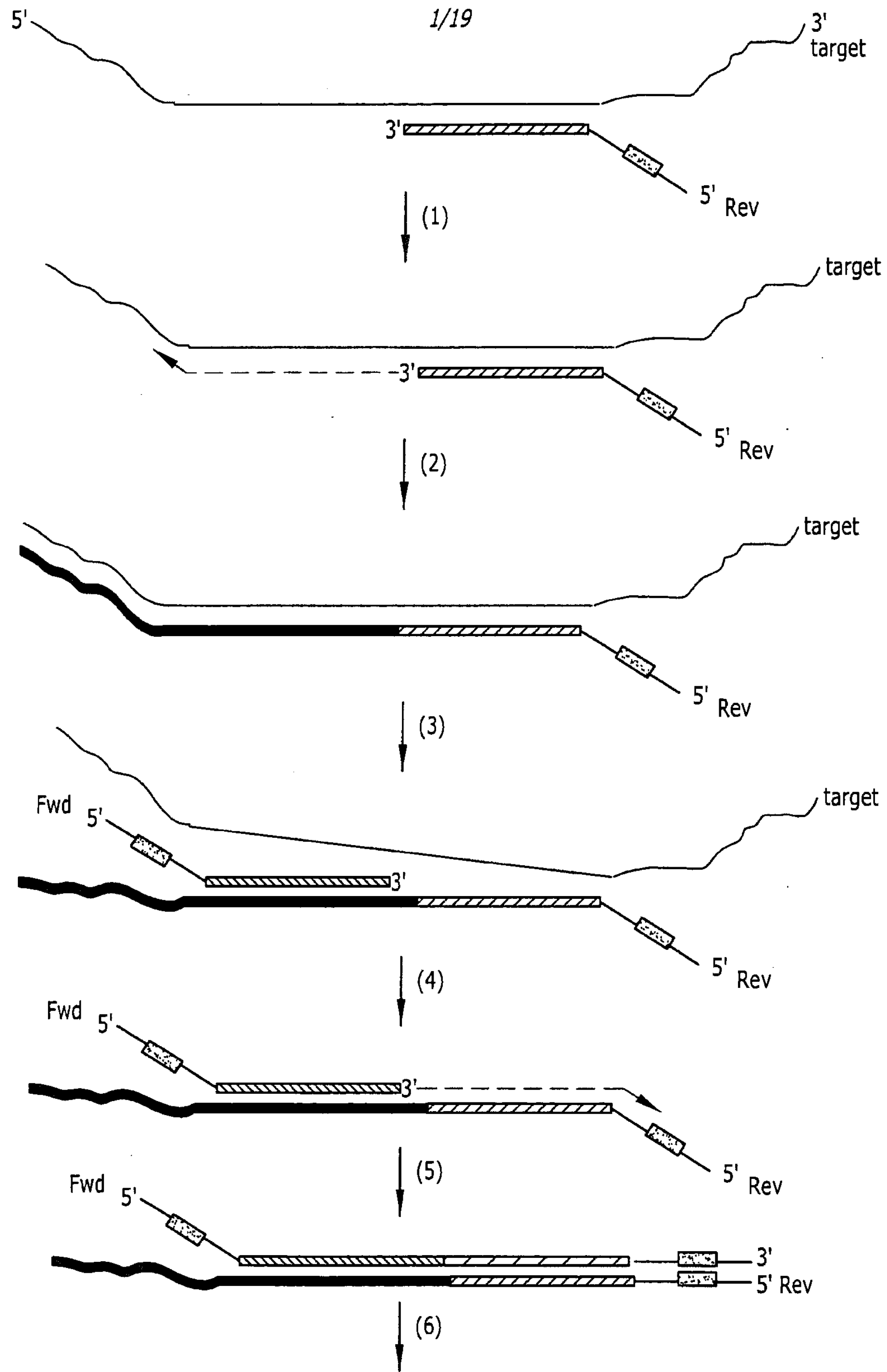


FIG. 1A

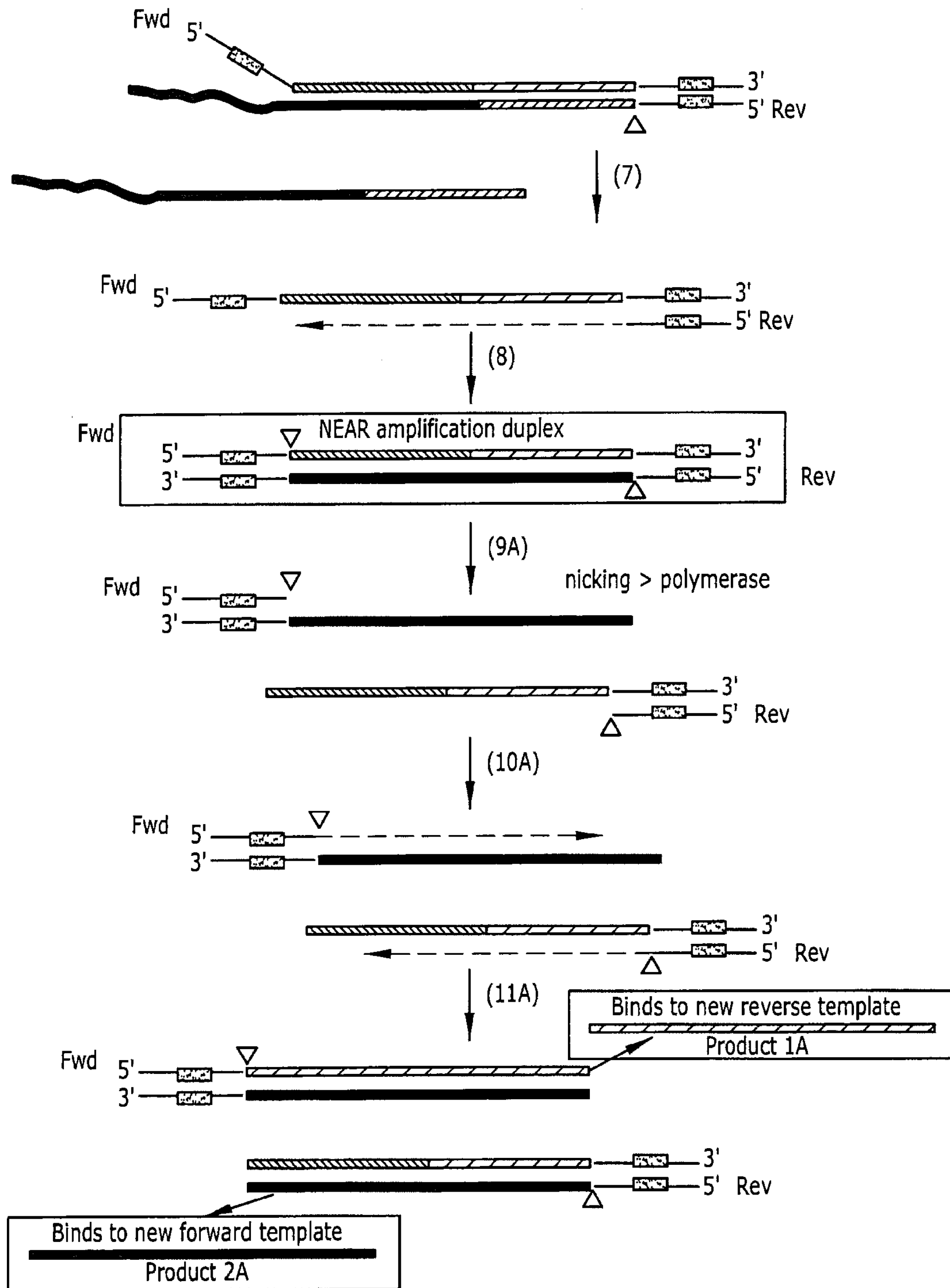


FIG. 1B

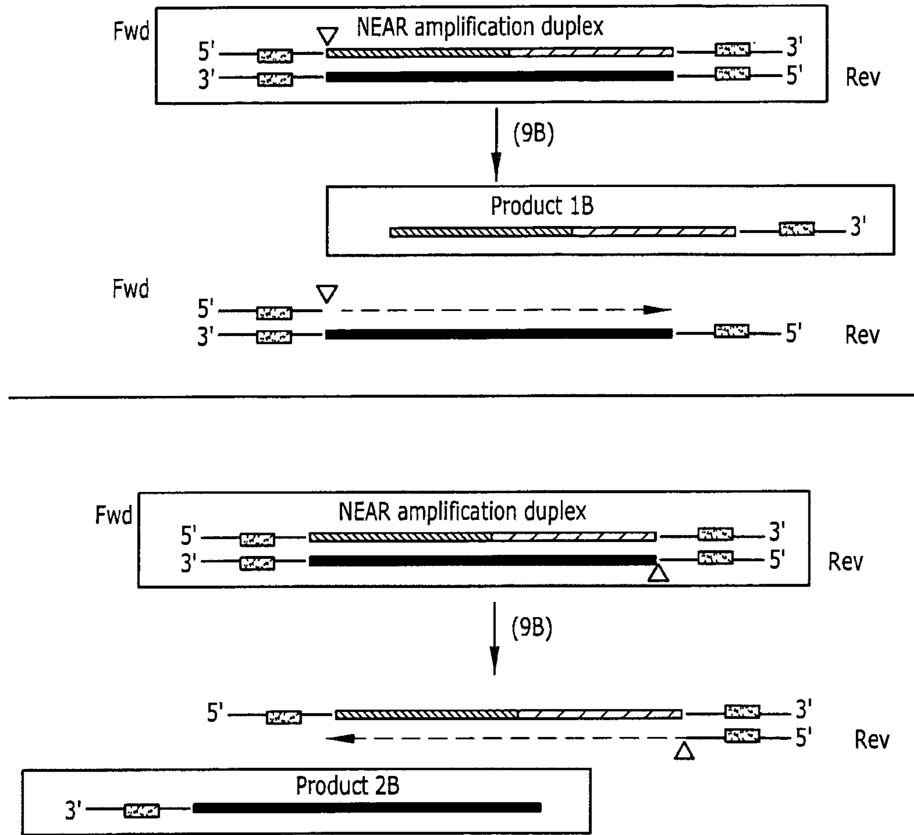


FIG. 1C

Recognition region to sense strand of target

Nicking Site

Nicking Site

Recognition site to antisense strand of target

FIG. 1D

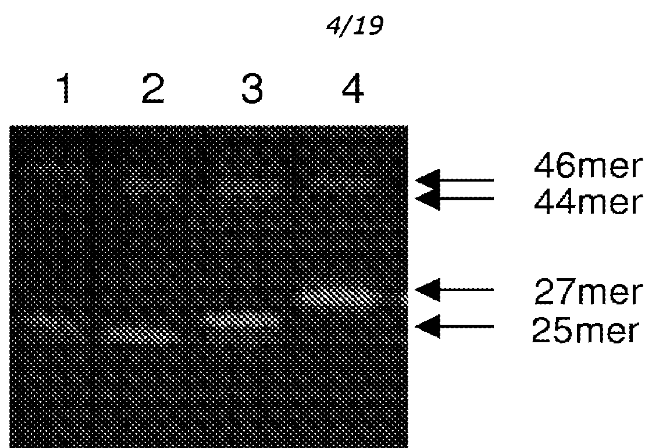


FIG. 2

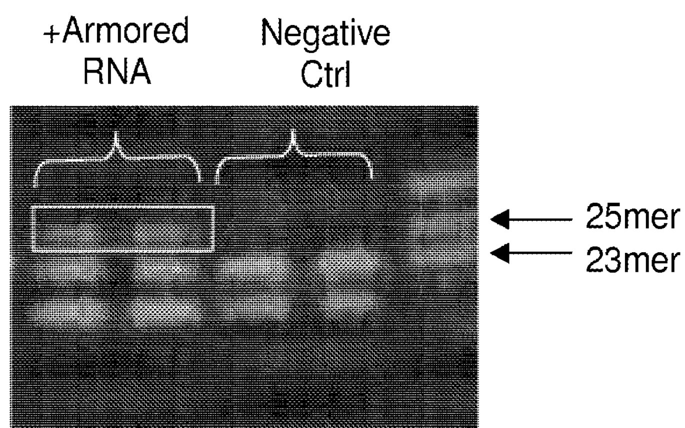


FIG. 3

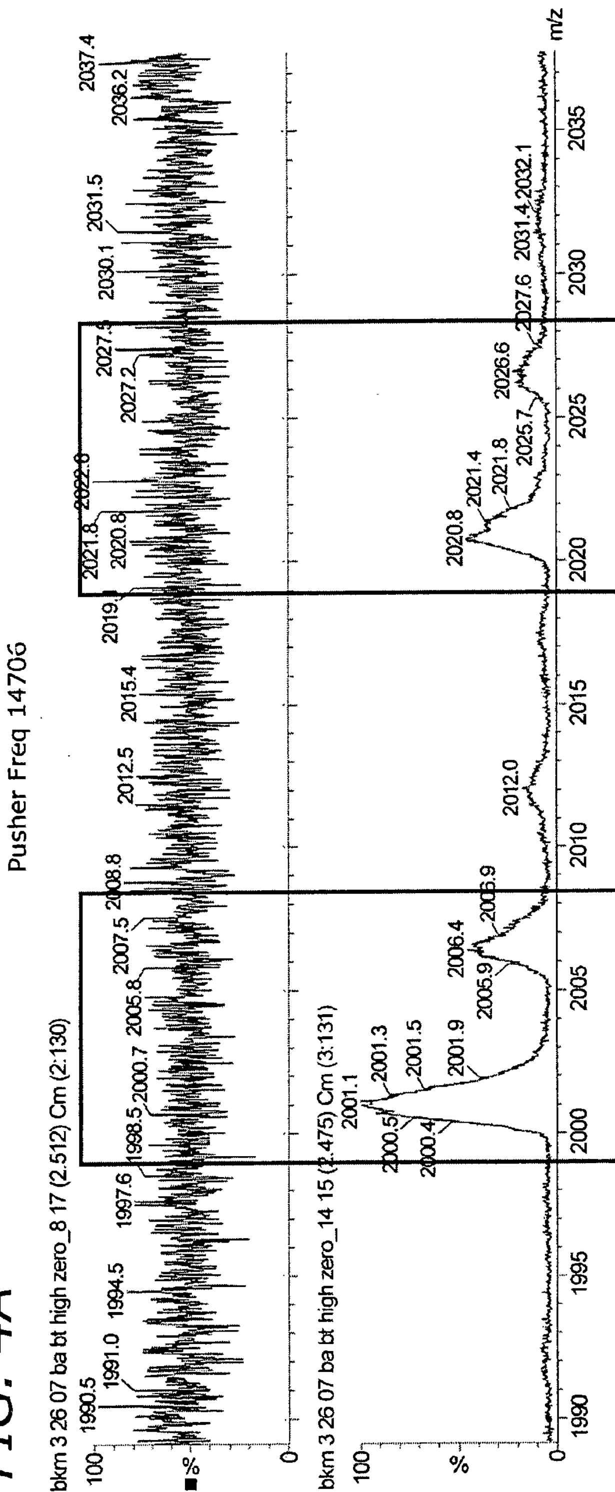
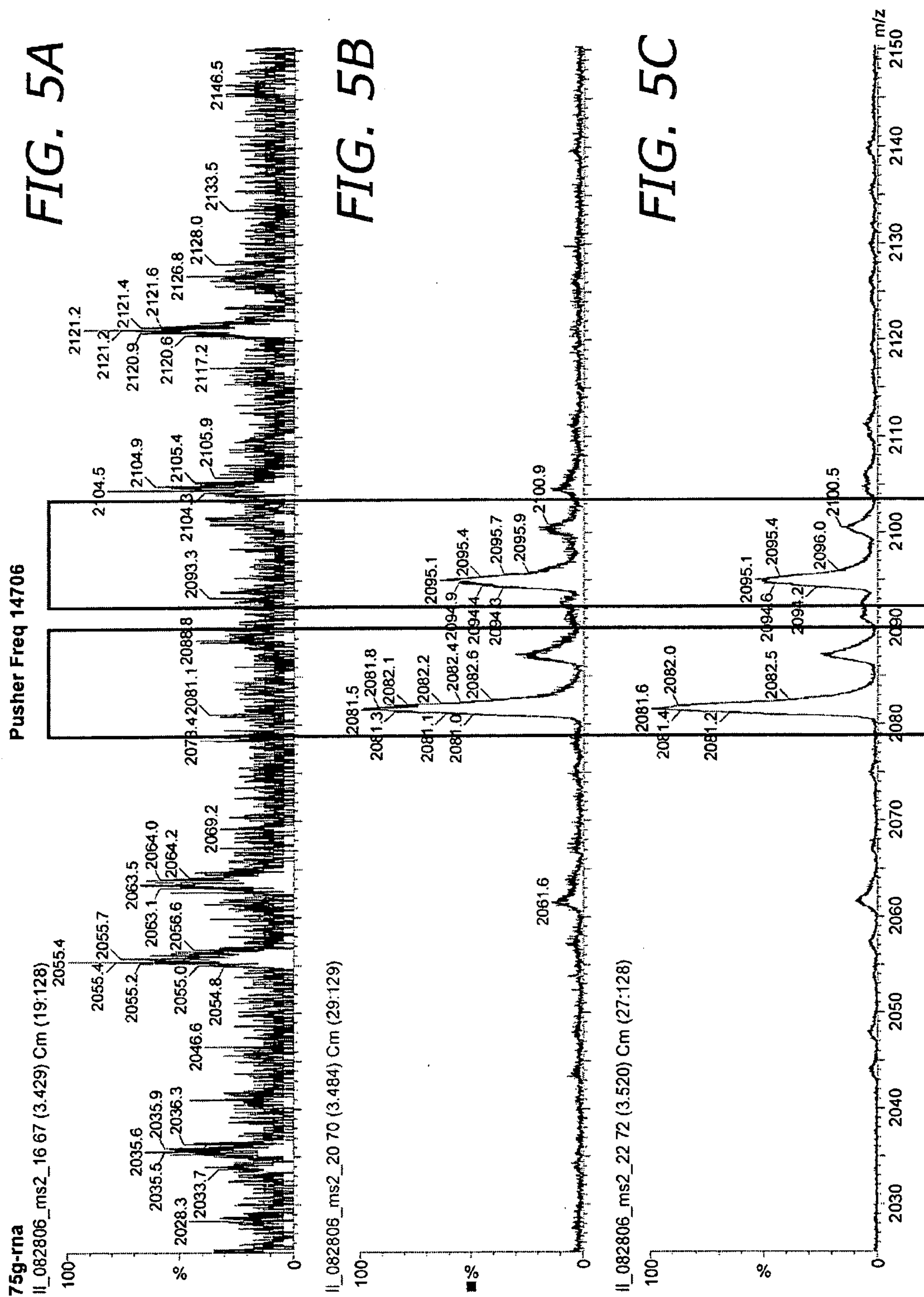


FIG. 4A

FIG. 4B


75g-rna
II_082806_ms2_16 67 (3.429) Cm (19:128)
Pusher Freq 14706
FIG. 5A
II_082806_ms2_20 70 (3.484) Cm (29:129)
FIG. 5B
II_082806_ms2_22 72 (3.520) Cm (27:128)
FIG. 5C
100
%
0
2030
2040
2050
2060
2070
2080
2090
2100
2110
2120
2130
2140
2150
m/z

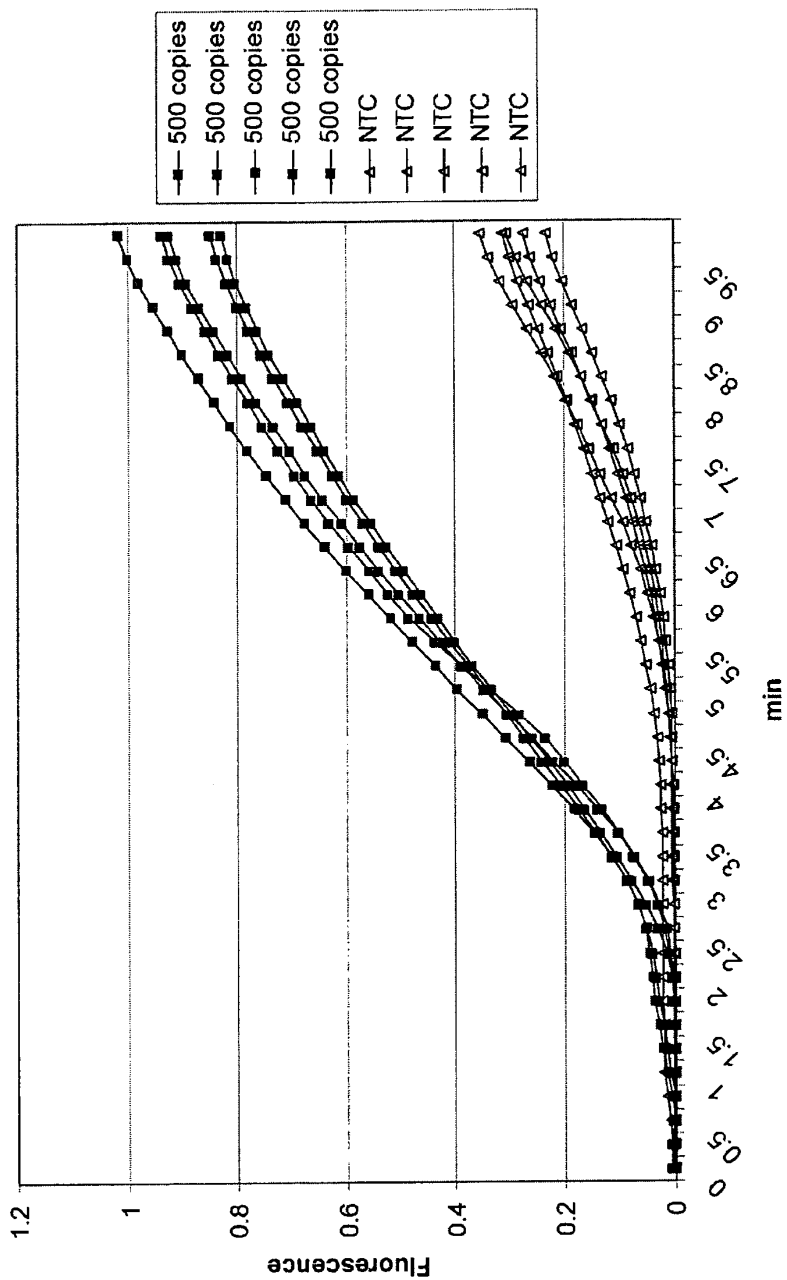


FIG. 6

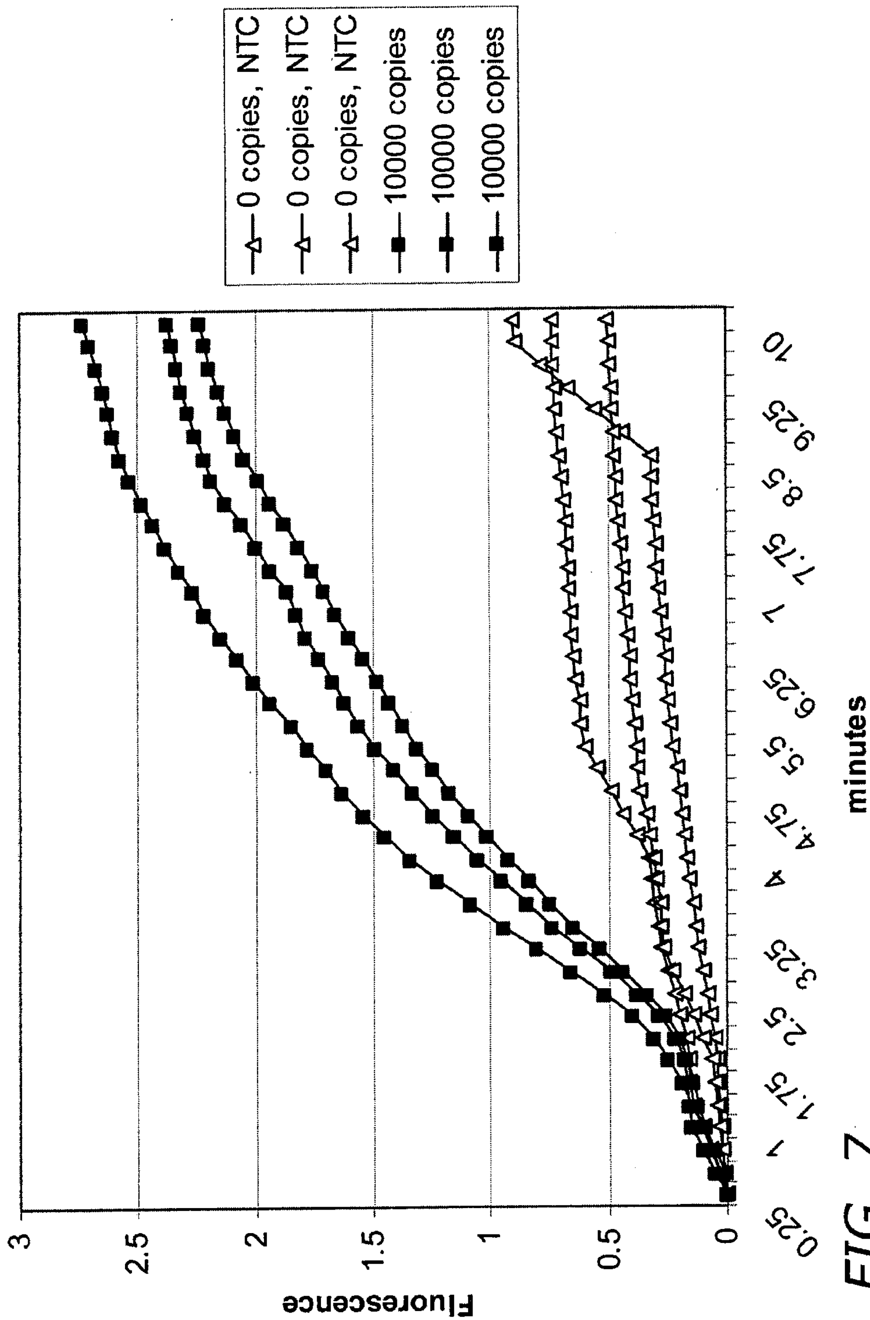


FIG. 7

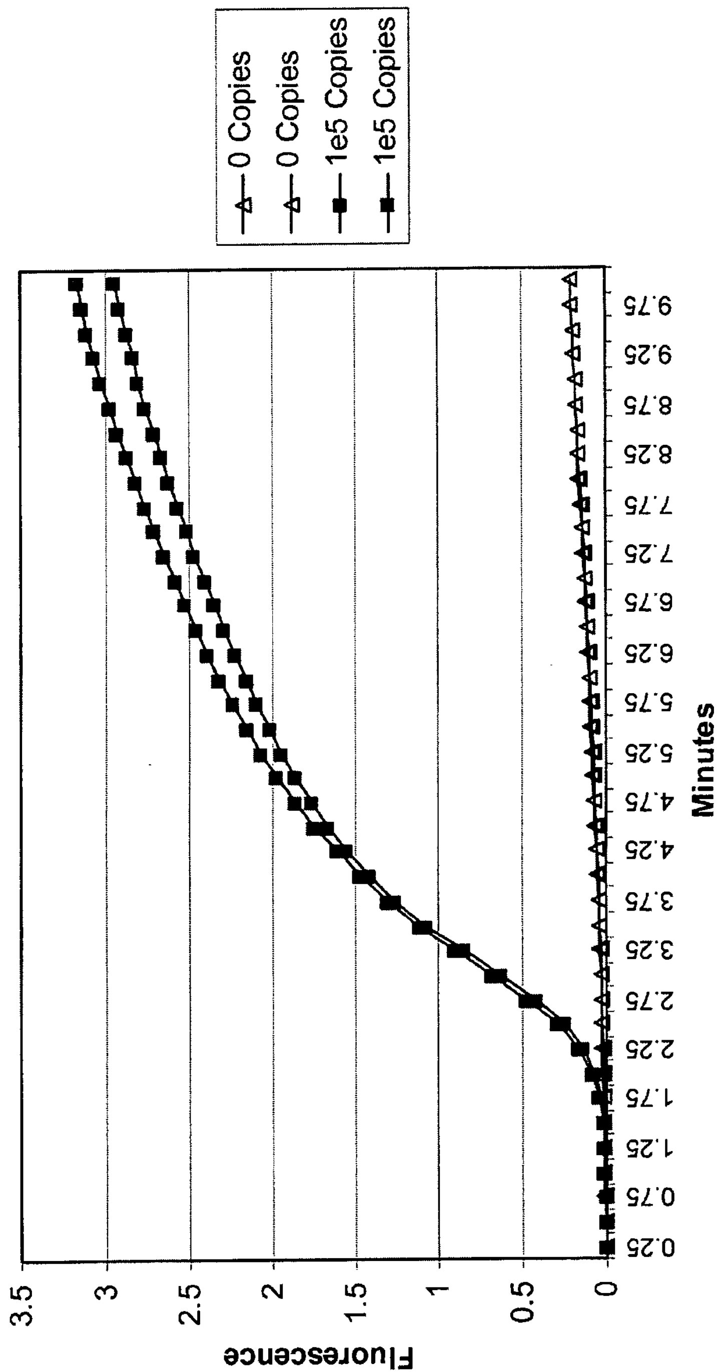


FIG. 8

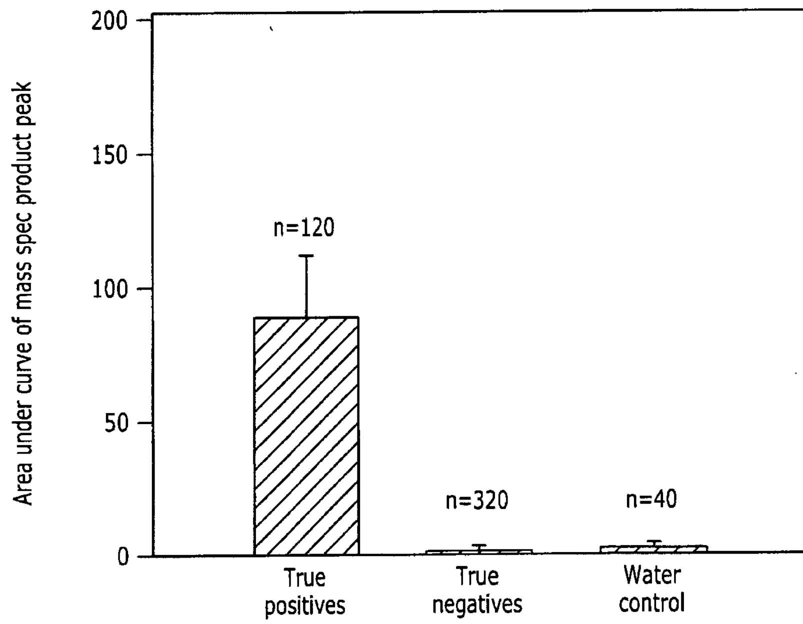


FIG. 9

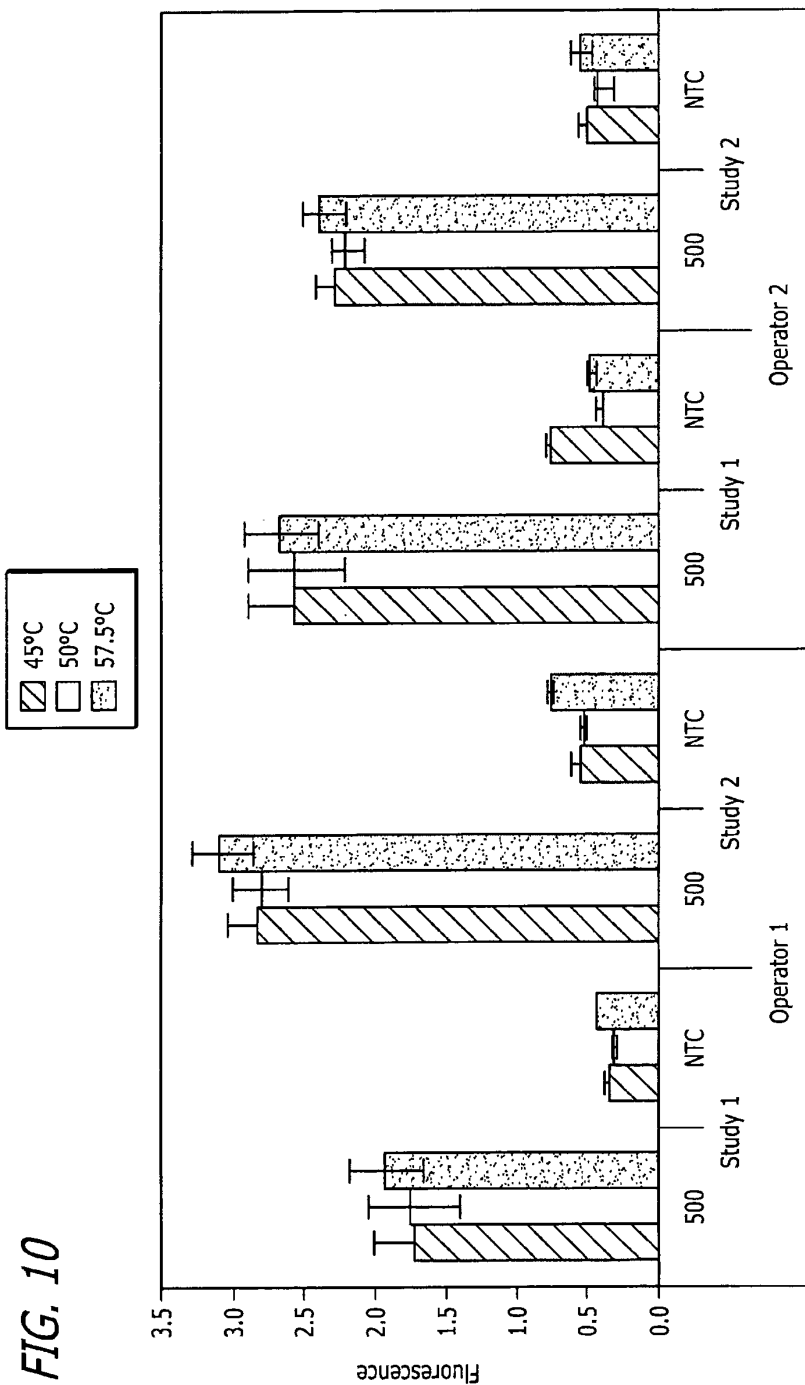


FIG. 10
Fluorescence
0.0
0.5
1.0
1.5
2.0
2.5
3.0
3.5
45°C
50°C
57.5°C
500
NTC
500
NTC
500
NTC
500
NTC
Study 1
Study 2
Study 1
Study 2
Operator 1
Operator 2

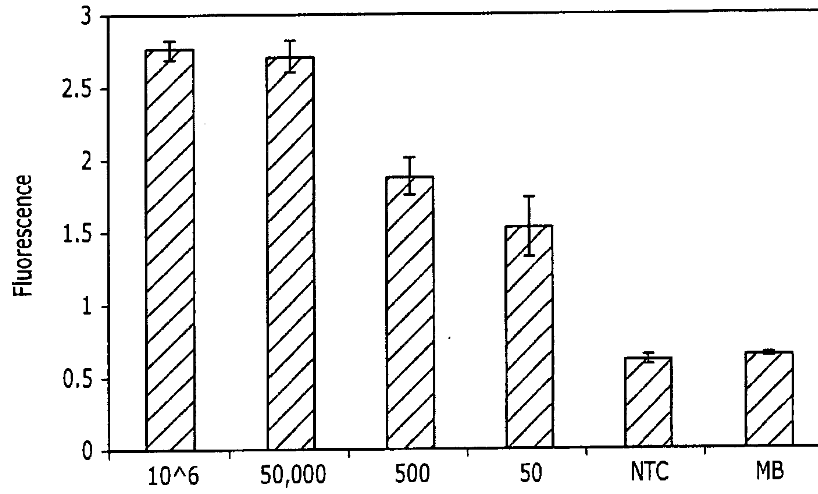


FIG. 11

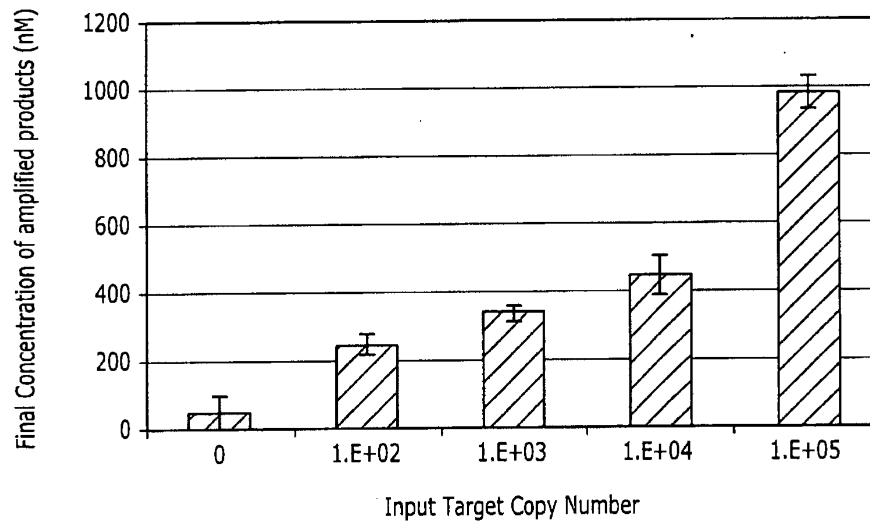


FIG. 12

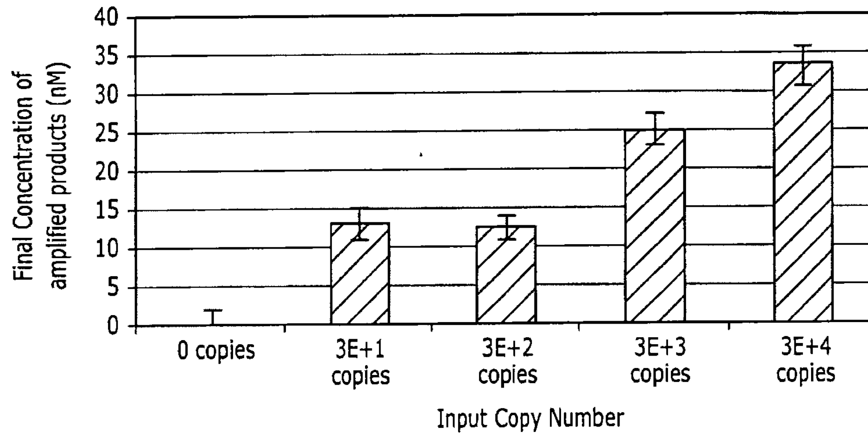


FIG. 13

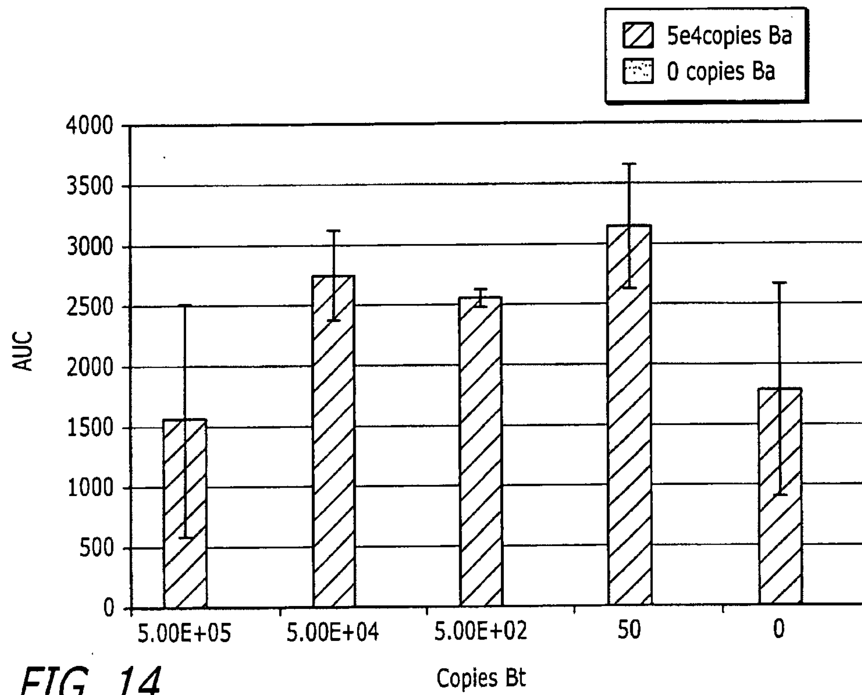


FIG. 14

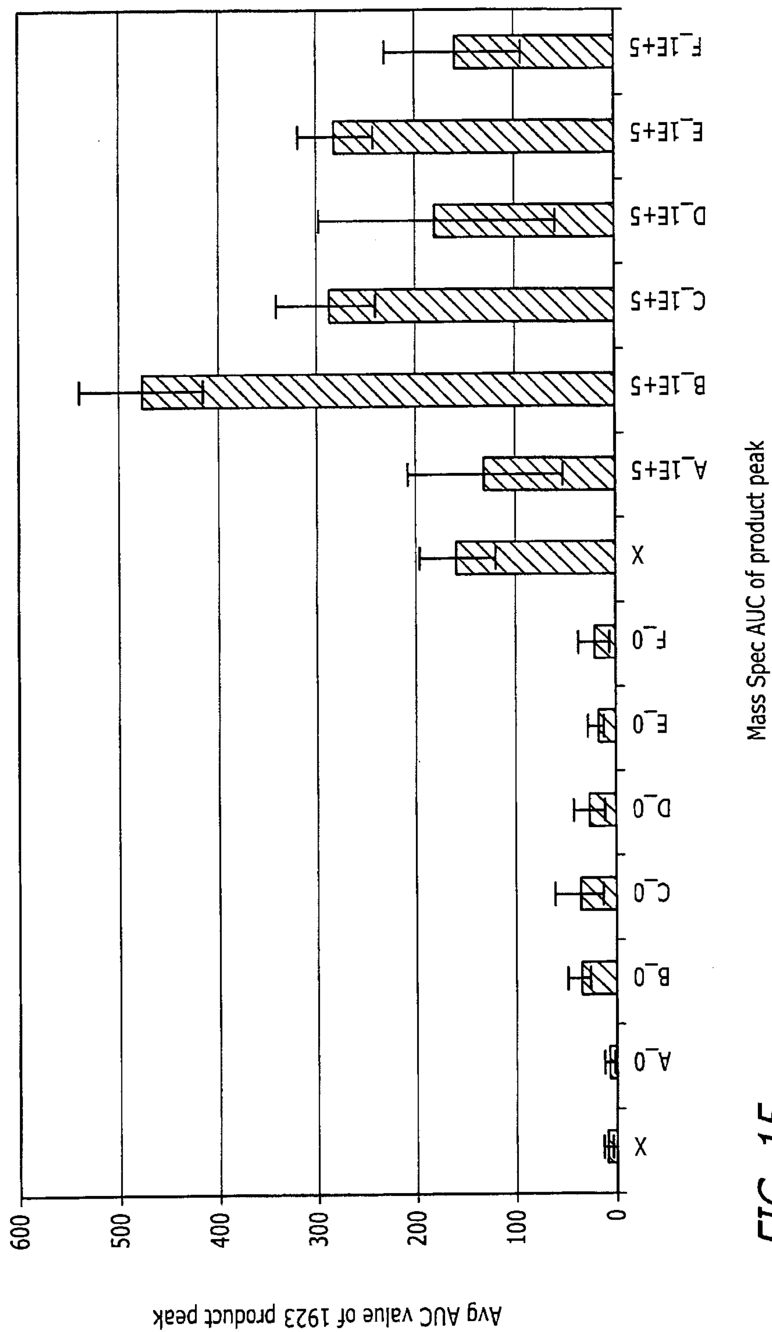


FIG. 15

FIG. 16

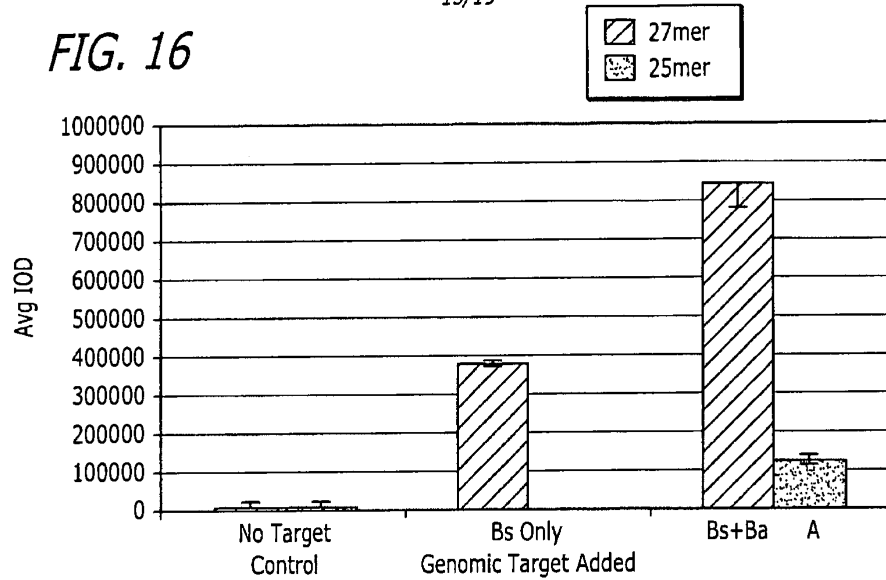
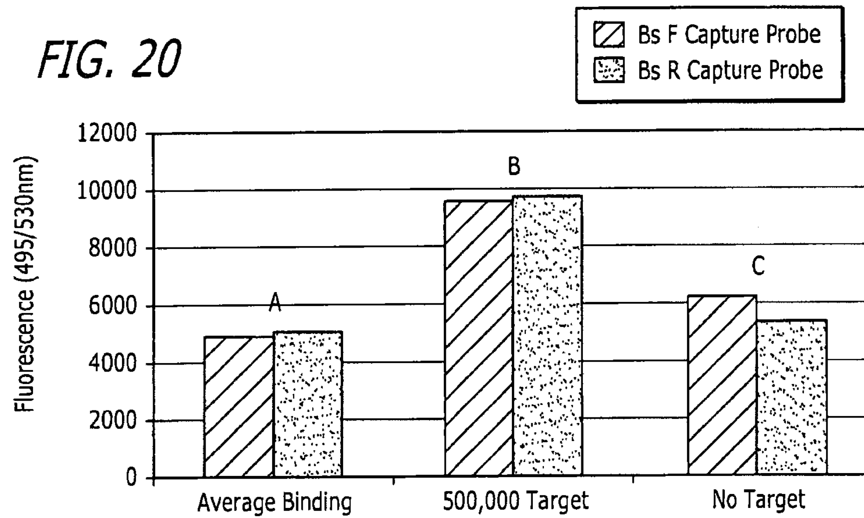


FIG. 20



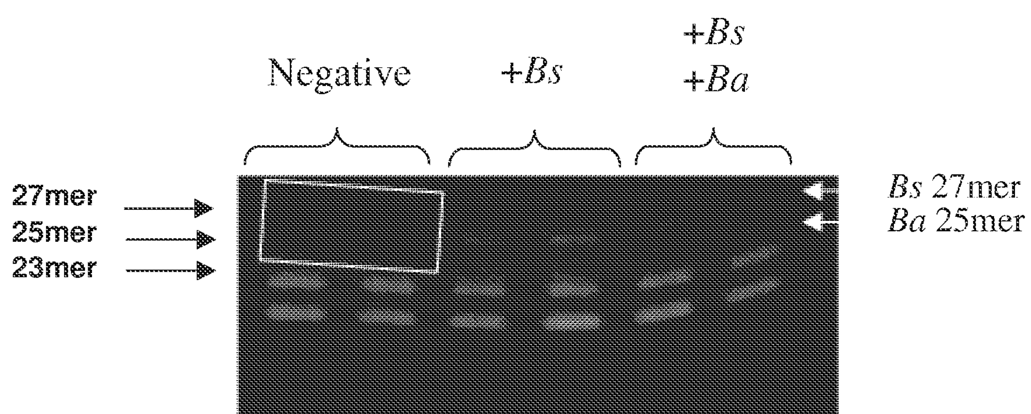


Figure 17

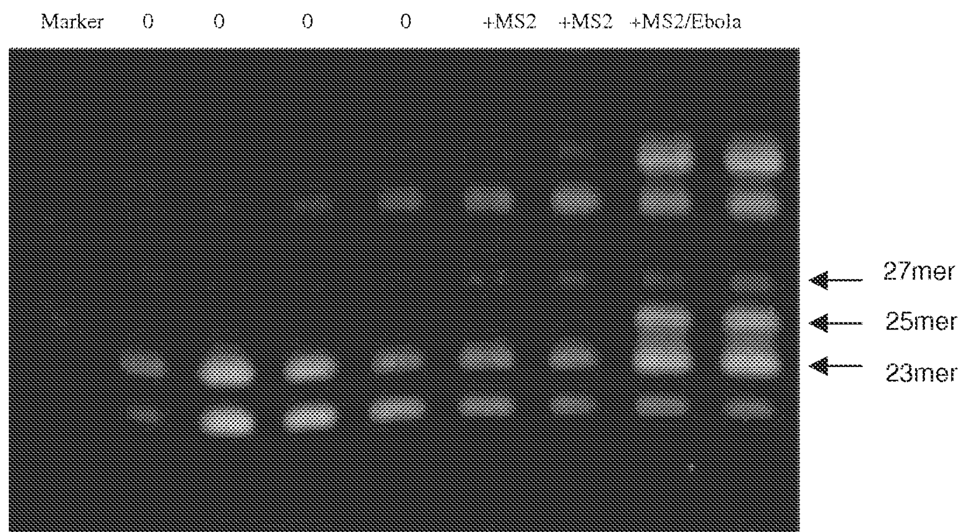


Figure 18

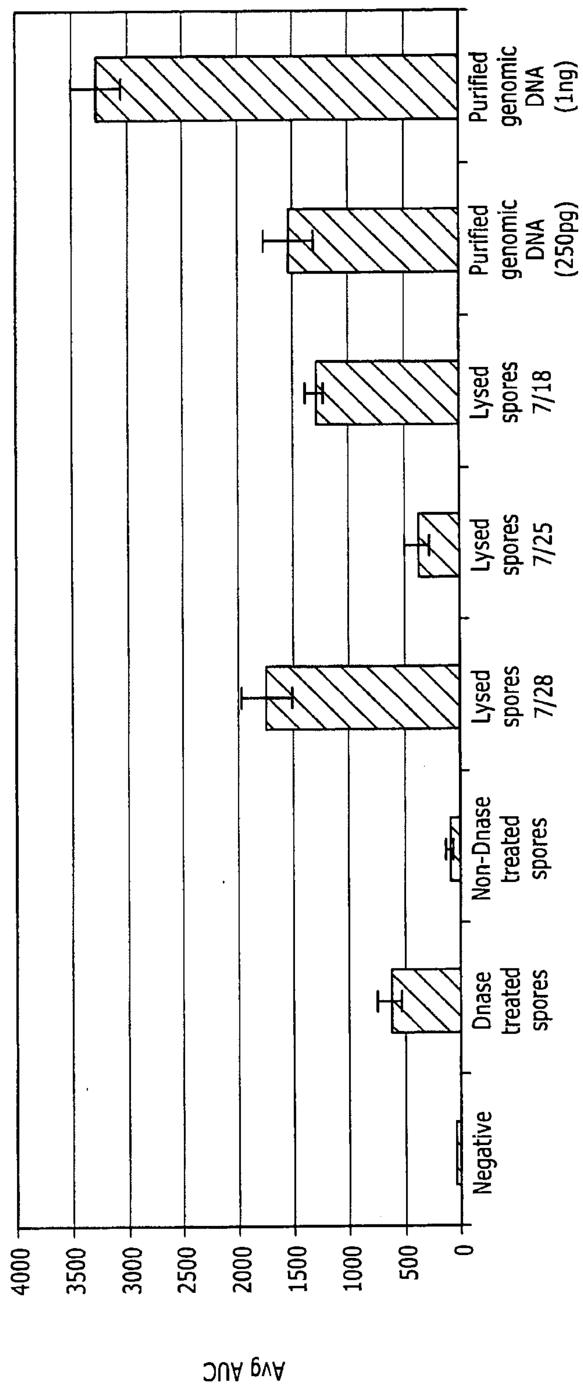


FIG. 19

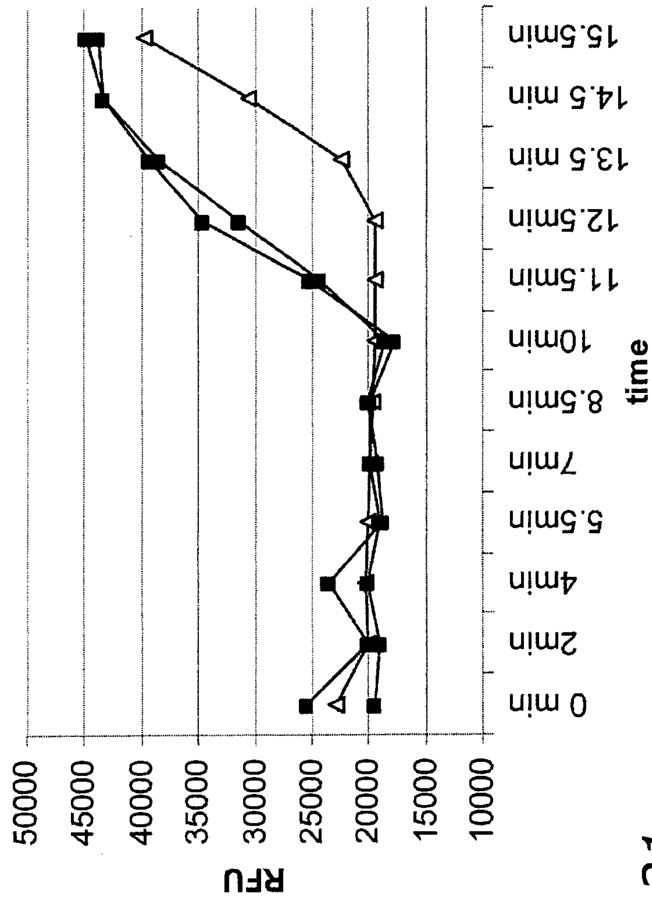


FIG. 21

Electronic Patent Application Fee Transmittal

Application Number:	14067620
Filing Date:	30-Oct-2013
Title of Invention:	Nicking and Extension Amplification Reaction for the Exponential Amplification of Nucleic Acids
First Named Inventor/Applicant Name:	Brian K. Maples
Filer:	Ian J.S. Lodovice/Mary Florczak
Attorney Docket Number:	30171-0025002 / ITI-001

Filed as Large Entity

Filing Fees for **Utility under 35 USC 111(a)**

Description	Fee Code	Quantity	Amount	Sub-Total in USD($)
Basic Filing:				
Pages:				
Claims:				
Miscellaneous-Filing:				
Petition:				
Patent-Appeals-and-Interference:				
Post-Allowance-and-Post-Issuance:				
Extension-of-Time:				

Description	Fee Code	Quantity	Amount	Sub-Total in USD($)
Miscellaneous:				
Submission- Information Disclosure Stmt	1806	1	180	180
		Total in USD ($)		**180**

Electronic Acknowledgement Receipt

EFS ID:	21172008
Application Number:	14067620
International Application Number:	
Confirmation Number:	4288
Title of Invention:	Nicking and Extension Amplification Reaction for the Exponential Amplification of Nucleic Acids
First Named Inventor/Applicant Name:	Brian K. Maples
Customer Number:	26161
Filer:	Ian J.S. Lodovice/Mary Florczak
Filer Authorized By:	Ian J.S. Lodovice
Attorney Docket Number:	30171-0025002 / ITI-001
Receipt Date:	09-JAN-2015
Filing Date:	30-OCT-2013
Time Stamp:	16:06:06
Application Type:	Utility under 35 USC 111(a)

Payment information:

Submitted with Payment	yes
Payment Type	Deposit Account
Payment was successfully received in RAM	$180
RAM confirmation Number	2601
Deposit Account	061050
Authorized User	

The Director of the USPTO is hereby authorized to charge indicated fees and credit any overpayment as follows:

File Listing:

Document Number	Document Description	File Name	File Size(Bytes)/ Message Digest	Multi Part /.zip	Pages (if appl.)
1	Information Disclosure Statement (IDS) Form (SB08)	301710025002IDS.pdf	157730 274f4372954ed0cf2c1118fcd1640e245ea6f194	no	2
Warnings:					
Information:					
This is not an USPTO supplied IDS fillable form					
2	Non Patent Literature	30171proprietorsresponse.pdf	445076 7db00831ae899fa599ca0dda447060a1bbe38199	no	36
Warnings:					
Information:					
3		301710025002Resp.pdf	239607 2992b26469da64f088cea24b5a701be15502c2ce	yes	14

Multipart Description/PDF files in .zip description

Document Description	Start	End
Amendment/Req. Reconsideration-After Non-Final Reject	1	1
Claims	2	7
Applicant Arguments/Remarks Made in an Amendment	8	14

Document Number	Document Description	File Name	File Size(Bytes)/ Message Digest	Multi Part /.zip	Pages (if appl.)
Warnings:					
Information:					
4	Drawings-only black and white line drawings	301710025002ReplacementFIGS.pdf	4208508 03c2f947d6bcc2719443f702f37bd68247220e0d	no	19
Warnings:					
Information:					
5	Fee Worksheet (SB06)	fee-info.pdf	30661 150fea8604ba6d2890d443a533ec89dbd398cf80	no	2
Warnings:					
Information:					
			Total Files Size (in bytes):	5081582	

This Acknowledgement Receipt evidences receipt on the noted date by the USPTO of the indicated documents, characterized by the applicant, and including page counts, where applicable. It serves as evidence of receipt similar to a Post Card, as described in MPEP 503.

New Applications Under 35 U.S.C. 111

If a new application is being filed and the application includes the necessary components for a filing date (see 37 CFR 1.53(b)-(d) and MPEP 506), a Filing Receipt (37 CFR 1.54) will be issued in due course and the date shown on this Acknowledgement Receipt will establish the filing date of the application.

National Stage of an International Application under 35 U.S.C. 371

If a timely submission to enter the national stage of an international application is compliant with the conditions of 35 U.S.C. 371 and other applicable requirements a Form PCT/DO/EO/903 indicating acceptance of the application as a national stage submission under 35 U.S.C. 371 will be issued in addition to the Filing Receipt, in due course.

New International Application Filed with the USPTO as a Receiving Office

If a new international application is being filed and the international application includes the necessary components for an international filing date (see PCT Article 11 and MPEP 1810), a Notification of the International Application Number and of the International Filing Date (Form PCT/RO/105) will be issued in due course, subject to prescriptions concerning national security, and the date shown on this Acknowledgement Receipt will establish the international filing date of the application.

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

First Named Inventor	:	Brian K. Maples	Art Unit	:	1637
Serial No.	:	14/067,620	Examiner	:	Olayinka A. Oyeyemi
Filed	:	October 30, 2013	Conf. No.	:	4288
Title	:	NICKING AND EXTENSION AMPLIFICATION REACTION FOR THE EXPONENTIAL AMPLIFICATION OF NUCLEIC ACIDS			

MAIL STOP AMENDMENT
Commissioner for Patents
P.O. Box 1450
Alexandria, VA 22313-1450

INFORMATION DISCLOSURE STATEMENT

Please consider the references listed on the enclosed PTO-SB-08 or Disclosure Form. Foreign patent documents and non-patent literature are enclosed; cited U.S. patents and patent application publications will be provided on request.

This statement is being filed after a first action on the merits, but before receipt of a final action or a notice of allowance. A late submission fee in the amount of $180, specified by 37 CFR §1.17(p), is being paid with this statement.

Apply any necessary charges or credits to deposit account 06-1050, referencing the above attorney docket number.

Respectfully submitted,

Date: January 9, 2015_______________

/Ian J.S. Lodovice, Reg. No. 59,749/__________
Ian J. Lodovice
Reg. No. 59,749

Customer Number 26161
Fish & Richardson P.C.
Telephone: (617) 956-5972
Facsimile: (877) 769-7945

23338838.doc

Substitute Disclosure Form	U.S. Department of Commerce Patent and Trademark Office	Attorney Docket No. 30171-0025002	Application No. 14/067,620
Information Disclosure Statement by Applicant (Use several sheets if necessary) (37 CFR §1.98(b))		Applicant Ionian Technologies Inc.	
		Filing Date October 30, 2013	Group Art Unit 1637

U.S. Patent Documents

Examiner Initial	Desig. ID	Document Number	Publication Date	Patentee	Class	Subclass	Filing Date If Appropriate
	1	7,270,981	9/18/2007	Armes et al.			
	2	7,399,590	7/15/2008	Piepenburg et al.			
	3						

Foreign Patent Documents or Published Foreign Patent Applications

Examiner Initial	Desig. ID	Document Number	Publication Date	Country or Patent Office	Class	Subclass	Translation	
							Yes	No
	4							

Other Documents (include Author, Title, Date, and Place of Publication)

Examiner Initial	Desig. ID	Document
	5	Response of Patentee to Opposition in corresponding EP Application No. 08781827.4 (EP 2 181 196), dated January 7, 2015, pages 1-36.

Examiner Signature	Date Considered
EXAMINER: Initials citation considered. Draw line through citation if not in conformance and not considered. Include copy of this form with next communication to applicant.	

Substitute Disclosure Form

PTO/SB/06 (09-11)
Approved for use through 1/31/2014. OMB 0651-0032
U.S. Patent and Trademark Office; U.S. DEPARTMENT OF COMMERCE
Under the Paperwork Reduction Act of 1995, no persons are required to respond to a collection of information unless it displays a valid OMB control number.

PATENT APPLICATION FEE DETERMINATION RECORD Substitute for Form PTO-875	Application or Docket Number 14/067,620	Filing Date 10/30/2013	☐ To be Mailed

ENTITY: ☒ LARGE ☐ SMALL ☐ MICRO

APPLICATION AS FILED – PART I

FOR	NUMBER FILED (Column 1)	NUMBER EXTRA (Column 2)	RATE ($)	FEE ($)
☐ BASIC FEE (37 CFR 1.16(a), (b), or (c))	N/A	N/A	N/A	
☐ SEARCH FEE (37 CFR 1.16(k), (i), or (m))	N/A	N/A	N/A	
☐ EXAMINATION FEE (37 CFR 1.16(o), (p), or (q))	N/A	N/A	N/A	
TOTAL CLAIMS (37 CFR 1.16(i))	minus 20 =	*	X $ =	
INDEPENDENT CLAIMS (37 CFR 1.16(h))	minus 3 =	*	X $ =	
☐ APPLICATION SIZE FEE (37 CFR 1.16(s))	If the specification and drawings exceed 100 sheets of paper, the application size fee due is $310 ($155 for small entity) for each additional 50 sheets or fraction thereof. See 35 U.S.C. 41(a)(1)(G) and 37 CFR 1.16(s).			
☐ MULTIPLE DEPENDENT CLAIM PRESENT (37 CFR 1.16(j))				
* If the difference in column 1 is less than zero, enter "0" in column 2.			TOTAL	

APPLICATION AS AMENDED – PART II

AMENDMENT	01/09/2015	CLAIMS REMAINING AFTER AMENDMENT (Column 1)		HIGHEST NUMBER PREVIOUSLY PAID FOR (Column 2)	PRESENT EXTRA (Column 3)	RATE ($)	ADDITIONAL FEE ($)
	Total (37 CFR 1.16(i))	* 26	Minus	** 29	= 0	x $80 =	0
	Independent (37 CFR 1.16(h))	* 3	Minus	***3	= 0	x $420 =	0
	☐ Application Size Fee (37 CFR 1.16(s))						
	☐ FIRST PRESENTATION OF MULTIPLE DEPENDENT CLAIM (37 CFR 1.16(j))						
						TOTAL ADD'L FEE	**0**

AMENDMENT		CLAIMS REMAINING AFTER AMENDMENT (Column 1)		HIGHEST NUMBER PREVIOUSLY PAID FOR (Column 2)	PRESENT EXTRA (Column 3)	RATE ($)	ADDITIONAL FEE ($)
	Total (37 CFR 1.16(i))	*	Minus	**	=	X $ =	
	Independent (37 CFR 1.16(h))	*	Minus	***	=	X $ =	
	☐ Application Size Fee (37 CFR 1.16(s))						
	☐ FIRST PRESENTATION OF MULTIPLE DEPENDENT CLAIM (37 CFR 1.16(j))						
						TOTAL ADD'L FEE	

* If the entry in column 1 is less than the entry in column 2, write "0" in column 3.
** If the "Highest Number Previously Paid For" IN THIS SPACE is less than 20, enter "20".
*** If the "Highest Number Previously Paid For" IN THIS SPACE is less than 3, enter "3".
The "Highest Number Previously Paid For" (Total or Independent) is the highest number found in the appropriate box in column 1.

LIE
/ROSALIND BALL/

This collection of information is required by 37 CFR 1.16. The information is required to obtain or retain a benefit by the public which is to file (and by the USPTO to process) an application. Confidentiality is governed by 35 U.S.C. 122 and 37 CFR 1.14. This collection is estimated to take 12 minutes to complete, including gathering, preparing, and submitting the completed application form to the USPTO. Time will vary depending upon the individual case. Any comments on the amount of time you require to complete this form and/or suggestions for reducing this burden, should be sent to the Chief Information Officer, U.S. Patent and Trademark Office, U.S. Department of Commerce, P.O. Box 1450, Alexandria, VA 22313-1450. DO NOT SEND FEES OR COMPLETED FORMS TO THIS ADDRESS. **SEND TO: Commissioner for Patents, P.O. Box 1450, Alexandria, VA 22313-1450.**
If you need assistance in completing the form, call 1-800-PTO-9199 and select option 2.

UNITED STATES PATENT AND TRADEMARK OFFICE

UNITED STATES DEPARTMENT OF COMMERCE
United States Patent and Trademark Office
Address: COMMISSIONER FOR PATENTS
P.O. Box 1450
Alexandria, Virginia 22313-1450
www.uspto.gov

APPLICATION NO.	FILING DATE	FIRST NAMED INVENTOR	ATTORNEY DOCKET NO.	CONFIRMATION NO.
14/067,620	10/30/2013	Brian K. Maples	30171-0025002 / ITI-001	4288

26161 7590 12/12/2014
FISH & RICHARDSON P.C. (BO)
P.O. BOX 1022
MINNEAPOLIS, MN 55440-1022

EXAMINER	
BERTAGNA, ANGELA MARIE	

ART UNIT	PAPER NUMBER
1637	

NOTIFICATION DATE	DELIVERY MODE
12/12/2014	ELECTRONIC

Please find below and/or attached an Office communication concerning this application or proceeding.

The time period for reply, if any, is set in the attached communication.

Notice of the Office communication was sent electronically on above-indicated "Notification Date" to the following e-mail address(es):

PATDOCTC@fr.com

PTOL-90A (Rev. 04/07)

Applicant-Initiated Interview Summary	**Application No.** 14/067,620	**Applicant(s)** MAPLES ET AL.	
	Examiner Angela M. Bertagna	**Art Unit** 1637	

All participants (applicant, applicant's representative, PTO personnel):

(1) *Angela M. Bertagna*.

(2) *Ian Lodovice (Applicant's rep)*.

(3) *Belinda Liu, Julius Fister, III (Applicant's reps)*.

(4) *Rich Roth (Applicant)*.

Date of Interview: *08 December 2014*.

Type: ☐ Telephonic ☐ Video Conference
☒ Personal [copy given to: ☐ applicant ☐ applicant's representative]

Exhibit shown or demonstration conducted: ☐ Yes ☒ No.
If Yes, brief description: ______.

Issues Discussed ☐101 ☐112 ☒102 ☒103 ☐Others
(For each of the checked box(es) above, please describe below the issue and detailed description of the discussion)

Claim(s) discussed: *67-73,76,77,80 and 83-94*.

Identification of prior art discussed: *Van Ness et al. (WO 2003/008642), Wick et al. (US 6,063,604), and Kong et al. (US 6,191,267), each of which was cited in the last office action*.

Substance of Interview
(For each issue discussed, provide a detailed description and indicate if agreement was reached. Some topics may include: identification or clarification of a reference or a portion thereof, claim interpretation, proposed amendments, arguments of any applied references etc...)

We discussed the nature of the invention and the differences between the invention and the cited references. We also discussed possible amendments to the claims that may distinguish them from the cited references. No specific agreement was reached.

Applicant recordation instructions: The formal written reply to the last Office action must include the substance of the interview. (See MPEP section 713.04). If a reply to the last Office action has already been filed, applicant is given a non-extendable period of the longer of one month or thirty days from this interview date, or the mailing date of this interview summary form, whichever is later, to file a statement of the substance of the interview

Examiner recordation instructions: Examiners must summarize the substance of any interview of record. A complete and proper recordation of the substance of an interview should include the items listed in MPEP 713.04 for complete and proper recordation including the identification of the general thrust of each argument or issue discussed, a general indication of any other pertinent matters discussed regarding patentability and the general results or outcome of the interview, to include an indication as to whether or not agreement was reached on the issues raised.

☐ Attachment

/Angela M. Bertagna/ Primary Examiner, Art Unit 1637	

Summary of Record of Interview Requirements

Manual of Patent Examining Procedure (MPEP), Section 713.04, Substance of Interview Must be Made of Record
A complete written statement as to the substance of any face-to-face, video conference, or telephone interview with regard to an application must be made of record in the application whether or not an agreement with the examiner was reached at the interview.

Title 37 Code of Federal Regulations (CFR) § 1.133 Interviews
Paragraph (b)

In every instance where reconsideration is requested in view of an interview with an examiner, a complete written statement of the reasons presented at the interview as warranting favorable action must be filed by the applicant. An interview does not remove the necessity for reply to Office action as specified in §§ 1.111, 1.135. (35 U.S.C. 132)

37 CFR §1.2 Business to be transacted in writing.
All business with the Patent or Trademark Office should be transacted in writing. The personal attendance of applicants or their attorneys or agents at the Patent and Trademark Office is unnecessary. The action of the Patent and Trademark Office will be based exclusively on the written record in the Office. No attention will be paid to any alleged oral promise, stipulation, or understanding in relation to which there is disagreement or doubt.

The action of the Patent and Trademark Office cannot be based exclusively on the written record in the Office if that record is itself incomplete through the failure to record the substance of interviews.

It is the responsibility of the applicant or the attorney or agent to make the substance of an interview of record in the application file, unless the examiner indicates he or she will do so. It is the examiner's responsibility to see that such a record is made and to correct material inaccuracies which bear directly on the question of patentability.

Examiners must complete an Interview Summary Form for each interview held where a matter of substance has been discussed during the interview by checking the appropriate boxes and filling in the blanks. Discussions regarding only procedural matters, directed solely to restriction requirements for which interview recordation is otherwise provided for in Section 812.01 of the Manual of Patent Examining Procedure, or pointing out typographical errors or unreadable script in Office actions or the like, are excluded from the interview recordation procedures below. Where the substance of an interview is completely recorded in an Examiners Amendment, no separate Interview Summary Record is required.

The Interview Summary Form shall be given an appropriate Paper No., placed in the right hand portion of the file, and listed on the "Contents" section of the file wrapper. In a personal interview, a duplicate of the Form is given to the applicant (or attorney or agent) at the conclusion of the interview. In the case of a telephone or video-conference interview, the copy is mailed to the applicant's correspondence address either with or prior to the next official communication. If additional correspondence from the examiner is not likely before an allowance or if other circumstances dictate, the Form should be mailed promptly after the interview rather than with the next official communication.

The Form provides for recordation of the following information:
- Application Number (Series Code and Serial Number)
- Name of applicant
- Name of examiner
- Date of interview
- Type of interview (telephonic, video-conference, or personal)
- Name of participant(s) (applicant, attorney or agent, examiner, other PTO personnel, etc.)
- An indication whether or not an exhibit was shown or a demonstration conducted
- An identification of the specific prior art discussed
- An indication whether an agreement was reached and if so, a description of the general nature of the agreement (may be by attachment of a copy of amendments or claims agreed as being allowable). Note: Agreement as to allowability is tentative and does not restrict further action by the examiner to the contrary.
- The signature of the examiner who conducted the interview (if Form is not an attachment to a signed Office action)

It is desirable that the examiner orally remind the applicant of his or her obligation to record the substance of the interview of each case. It should be noted, however, that the Interview Summary Form will not normally be considered a complete and proper recordation of the interview unless it includes, or is supplemented by the applicant or the examiner to include, all of the applicable items required below concerning the substance of the interview.

A complete and proper recordation of the substance of any interview should include at least the following applicable items:
1) A brief description of the nature of any exhibit shown or any demonstration conducted,
2) an identification of the claims discussed,
3) an identification of the specific prior art discussed,
4) an identification of the principal proposed amendments of a substantive nature discussed, unless these are already described on the Interview Summary Form completed by the Examiner,
5) a brief identification of the general thrust of the principal arguments presented to the examiner,
   (The identification of arguments need not be lengthy or elaborate. A verbatim or highly detailed description of the arguments is not required. The identification of the arguments is sufficient if the general nature or thrust of the principal arguments made to the examiner can be understood in the context of the application file. Of course, the applicant may desire to emphasize and fully describe those arguments which he or she feels were or might be persuasive to the examiner.)
6) a general indication of any other pertinent matters discussed, and
7) if appropriate, the general results or outcome of the interview unless already described in the Interview Summary Form completed by the examiner.

Examiners are expected to carefully review the applicant's record of the substance of an interview. If the record is not complete and accurate, the examiner will give the applicant an extendable one month time period to correct the record.

Examiner to Check for Accuracy

If the claims are allowable for other reasons of record, the examiner should send a letter setting forth the examiner's version of the statement attributed to him or her. If the record is complete and accurate, the examiner should place the indication, "Interview Record OK" on the paper recording the substance of the interview along with the date and the examiner's initials.

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

First Named Inventor	:	Brian K. Maples	Art Unit	:	1637
Serial No.	:	14/067,620	Examiner	:	Olayinka A. Oyeyemi
Filed	:	October 30, 2013	Conf. No.	:	4288
Title	:	NICKING AND EXTENSION AMPLIFICATION REACTION FOR THE EXPONENTIAL AMPLIFICATION OF NUCLEIC ACIDS			

MAIL STOP AMENDMENT
Commissioner for Patents
P.O. Box 1450
Alexandria, VA 22313-1450

INFORMATION DISCLOSURE STATEMENT

Please consider the references listed on the enclosed PTO-SB-08 or Disclosure Form. Non-patent literature is enclosed.

This statement is being filed after a first action on the merits, but before receipt of a final action or a notice of allowance. A late submission fee in the amount of $180, specified by 37 CFR §1.17(p), is being paid with this statement.

Apply any necessary charges or credits to deposit account 06-1050, referencing the above attorney docket number.

Respectfully submitted,

Date: December 4, 2014

/Ian J.S. Lodovice, Reg. No. 59,749/
Ian J. Lodovice
Reg. No. 59,749

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23328165.doc

Substitute Disclosure Form U.S. Department of Commerce Patent and Trademark Office	Attorney Docket No. 30171-0025002	Application No. 14/067,620
Information Disclosure Statement by Applicant (Use several sheets if necessary) (37 CFR §1.98(b))	Applicant Ionian Technologies Inc.	
	Filing Date October 30, 2013	Group Art Unit 1637

U.S. Patent Documents

Examiner Initial	Desig. ID	Document Number	Publication Date	Patentee	Class	Subclass	Filing Date If Appropriate
	1						

Foreign Patent Documents or Published Foreign Patent Applications

Examiner Initial	Desig. ID	Document Number	Publication Date	Country or Patent Office	Class	Subclass	Translation Yes	Translation No
	2							

Other Documents (include Author, Title, Date, and Place of Publication)

Examiner Initial	Desig. ID	Document
	3	Walker et al., "Isothermal *in vitro* amplification of DNA by a restriction enzyme/DNA polymerase system," PNAS, 89:392-396 (1992)
	4	

Examiner Signature	Date Considered
EXAMINER: Initials citation considered. Draw line through citation if not in conformance and not considered. Include copy of this form with next communication to applicant.	

Substitute Disclosure Form

Electronic Patent Application Fee Transmittal

Application Number:	14067620
Filing Date:	30-Oct-2013
Title of Invention:	Nicking and Extension Amplification Reaction for the Exponential Amplification of Nucleic Acids
First Named Inventor/Applicant Name:	Brian K. Maples
Filer:	Ian J.S. Lodovice/Mary Florczak
Attorney Docket Number:	30171-0025002 / ITI-001

Filed as Large Entity

Utility under 35 USC 111(a) Filing Fees

Description	Fee Code	Quantity	Amount	Sub-Total in USD($)
Basic Filing:				
Pages:				
Claims:				
Miscellaneous-Filing:				
Petition:				
Patent-Appeals-and-Interference:				
Post-Allowance-and-Post-Issuance:				
Extension-of-Time:				

Description	Fee Code	Quantity	Amount	Sub-Total in USD($)
Miscellaneous:				
Submission- Information Disclosure Stmt	1806	1	180	180
		Total in USD ($)		**180**

Electronic Acknowledgement Receipt

EFS ID:	20880397
Application Number:	14067620
International Application Number:	
Confirmation Number:	4288
Title of Invention:	Nicking and Extension Amplification Reaction for the Exponential Amplification of Nucleic Acids
First Named Inventor/Applicant Name:	Brian K. Maples
Customer Number:	26161
Filer:	Ian J.S. Lodovice/Mary Florczak
Filer Authorized By:	Ian J.S. Lodovice
Attorney Docket Number:	30171-0025002 / ITI-001
Receipt Date:	05-DEC-2014
Filing Date:	30-OCT-2013
Time Stamp:	15:24:11
Application Type:	Utility under 35 USC 111(a)

Payment information:

Submitted with Payment	yes
Payment Type	Deposit Account
Payment was successfully received in RAM	$180
RAM confirmation Number	1792
Deposit Account	061050
Authorized User	

File Listing:

Document Number	Document Description	File Name	File Size(Bytes)/ Message Digest	Multi Part /.zip	Pages (if appl.)

1	Information Disclosure Statement (IDS) Form (SB08)	0025002IDS.pdf	170752 5f8e1e4fe3e27c6fd7d54db3cef35ba90fccfe4b	no	2
Warnings:					
Information:					
This is not an USPTO supplied IDS fillable form					
2	Non Patent Literature	30171Walker.pdf	1311886 d8cde0ef97e43e6b8aef4367282677525494c8e2	no	5
Warnings:					
Information:					
3	Fee Worksheet (SB06)	fee-info.pdf	30406 dca84188eb922bb54e4b1b78d938eb61a9246478	no	2
Warnings:					
Information:					
		Total Files Size (in bytes):	1513044		

This Acknowledgement Receipt evidences receipt on the noted date by the USPTO of the indicated documents, characterized by the applicant, and including page counts, where applicable. It serves as evidence of receipt similar to a Post Card, as described in MPEP 503.

New Applications Under 35 U.S.C. 111
If a new application is being filed and the application includes the necessary components for a filing date (see 37 CFR 1.53(b)-(d) and MPEP 506), a Filing Receipt (37 CFR 1.54) will be issued in due course and the date shown on this Acknowledgement Receipt will establish the filing date of the application.

National Stage of an International Application under 35 U.S.C. 371
If a timely submission to enter the national stage of an international application is compliant with the conditions of 35 U.S.C. 371 and other applicable requirements a Form PCT/DO/EO/903 indicating acceptance of the application as a national stage submission under 35 U.S.C. 371 will be issued in addition to the Filing Receipt, in due course.

New International Application Filed with the USPTO as a Receiving Office
If a new international application is being filed and the international application includes the necessary components for an international filing date (see PCT Article 11 and MPEP 1810), a Notification of the International Application Number and of the International Filing Date (Form PCT/RO/105) will be issued in due course, subject to prescriptions concerning national security, and the date shown on this Acknowledgement Receipt will establish the international filing date of the application.

UNITED STATES PATENT AND TRADEMARK OFFICE

UNITED STATES DEPARTMENT OF COMMERCE
United States Patent and Trademark Office
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Alexandria, Virginia 22313-1450
www.uspto.gov

APPLICATION NO.	FILING DATE	FIRST NAMED INVENTOR	ATTORNEY DOCKET NO.	CONFIRMATION NO.
14/067,620	10/30/2013	Brian K. Maples	30171-0025002 / ITI-001	4288

26161 7590 10/20/2014
FISH & RICHARDSON P.C. (BO)
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MINNEAPOLIS, MN 55440-1022

EXAMINER	
BERTAGNA, ANGELA MARIE	

ART UNIT	PAPER NUMBER
1637	

NOTIFICATION DATE	DELIVERY MODE
10/20/2014	ELECTRONIC

Please find below and/or attached an Office communication concerning this application or proceeding.

The time period for reply, if any, is set in the attached communication.

Notice of the Office communication was sent electronically on above-indicated "Notification Date" to the following e-mail address(es):

PATDOCTC@fr.com

PTOL-90A (Rev. 04/07)

Office Action Summary	**Application No.** 14/067,620	**Applicant(s)** MAPLES ET AL.	
	Examiner Angela M. Bertagna	**Art Unit** 1637	**AIA (First Inventor to File) Status** No

-- The MAILING DATE of this communication appears on the cover sheet with the correspondence address --

Period for Reply

A SHORTENED STATUTORY PERIOD FOR REPLY IS SET TO EXPIRE *3* MONTHS FROM THE MAILING DATE OF THIS COMMUNICATION.

- Extensions of time may be available under the provisions of 37 CFR 1.136(a). In no event, however, may a reply be timely filed after SIX (6) MONTHS from the mailing date of this communication.
- If NO period for reply is specified above, the maximum statutory period will apply and will expire SIX (6) MONTHS from the mailing date of this communication.
- Failure to reply within the set or extended period for reply will, by statute, cause the application to become ABANDONED (35 U.S.C. § 133). Any reply received by the Office later than three months after the mailing date of this communication, even if timely filed, may reduce any earned patent term adjustment. See 37 CFR 1.704(b).

Status

1)☒ Responsive to communication(s) filed on *4/2/14*.

☐ A declaration(s)/affidavit(s) under **37 CFR 1.130(b)** was/were filed on _____.

2a)☐ This action is **FINAL**. 2b)☒ This action is non-final.

3)☐ An election was made by the applicant in response to a restriction requirement set forth during the interview on _____; the restriction requirement and election have been incorporated into this action.

4)☐ Since this application is in condition for allowance except for formal matters, prosecution as to the merits is closed in accordance with the practice under *Ex parte Quayle*, 1935 C.D. 11, 453 O.G. 213.

Disposition of Claims*

5)☒ Claim(s) *67-95* is/are pending in the application.

5a) Of the above claim(s) *74,75,78,79,81,82 and 95* is/are withdrawn from consideration.

6)☐ Claim(s) _____ is/are allowed.

7)☒ Claim(s) *67-73,76,77,80 and 83-94* is/are rejected.

8)☐ Claim(s) _____ is/are objected to.

9)☐ Claim(s) _____ are subject to restriction and/or election requirement.

* If any claims have been determined allowable, you may be eligible to benefit from the **Patent Prosecution Highway** program at a participating intellectual property office for the corresponding application. For more information, please see http://www.uspto.gov/patents/init_events/pph/index.jsp or send an inquiry to PPHfeedback@uspto.gov.

Application Papers

10)☒ The specification is objected to by the Examiner.

11)☒ The drawing(s) filed on *12/18/13* is/are: a)☐ accepted or b)☒ objected to by the Examiner.

Applicant may not request that any objection to the drawing(s) be held in abeyance. See 37 CFR 1.85(a).

Replacement drawing sheet(s) including the correction is required if the drawing(s) is objected to. See 37 CFR 1.121(d).

Priority under 35 U.S.C. § 119

12)☐ Acknowledgment is made of a claim for foreign priority under 35 U.S.C. § 119(a)-(d) or (f).

Certified copies:

a)☐ All b)☐ Some** c)☐ None of the:

1.☐ Certified copies of the priority documents have been received.

2.☐ Certified copies of the priority documents have been received in Application No. _____.

3.☐ Copies of the certified copies of the priority documents have been received in this National Stage application from the International Bureau (PCT Rule 17.2(a)).

** See the attached detailed Office action for a list of the certified copies not received.

Attachment(s)

1) ☐ Notice of References Cited (PTO-892)

2) ☒ Information Disclosure Statement(s) (PTO/SB/08a and/or PTO/SB/08b) Paper No(s)/Mail Date *6/26/14; 5/23/14; 10/30/13*.

3) ☐ Interview Summary (PTO-413) Paper No(s)/Mail Date. _____ .

4) ☐ Other: _____.

DETAILED ACTION

Notice of Pre-AIA or AIA Status

1. The present application is being examined under the pre-AIA first to invent provisions.

Election/Restrictions

2. Applicant's response to the election of species requirement filed on April 2, 2014 is acknowledged. The species "single-stranded RNA virus as the target nucleic acid" was elected without traverse.

Applicant states that claims 67-73, 76, 77, 80, and 83-95 read on the elected species. This is not correct because claim 95 states that the target nucleic acid sequence is contained in genomic DNA. Only claims 67-73, 76, 77, 80, and 83-94 read on the elected species.

Claims 74, 75, 78, 79, 81, 82, and 95 are withdrawn from further consideration pursuant to 37 CFR 1.142(b) as being drawn to a nonelected species, there being no allowable generic or linking claim. Election was made **without** traverse in the reply filed on April 2, 2014.

Drawings

3. The submission of replacement drawing sheets on December 18, 2013 is acknowledged. These drawings are not acceptable because Figures 17 and 18 are not labeled as "Replacement Sheet". See 37 CFR 1.121(d).

Specification

4. The substitute specification filed on December 18, 2013 has been entered.

The specification is objected to as failing to provide proper antecedent basis for the claimed subject matter. See 37 CFR 1.75(d)(1) and MPEP § 608.01(o). Correction of the following is required: The specification does not provide proper antecedent basis for the subject matter of claim 93.

Claim Rejections - 35 USC § 112

5. The following is a quotation of 35 U.S.C. 112(b):

(b) CONCLUSION.—The specification shall conclude with one or more claims particularly pointing out and distinctly claiming the subject matter which the inventor or a joint inventor regards as the invention.

The following is a quotation of 35 U.S.C. 112 (pre-AIA), second paragraph:

The specification shall conclude with one or more claims particularly pointing out and distinctly claiming the subject matter which the applicant regards as his invention.

Claims 76, 77, 84, and 87-94 are rejected under 35 U.S.C. 112(b) or 35 U.S.C. 112 (pre-AIA), second paragraph, as being indefinite for failing to particularly point out and distinctly claim the subject matter which the inventor or a joint inventor, or for pre-AIA the applicant regards as the invention.

Regarding claim 76, the phrase "for example" renders the claim indefinite because it is unclear whether the limitation(s) following the phrase are part of the claimed invention. See MPEP § 2173.05(d).

Claim 77 is indefinite by way of its dependency on claim 76.

Claims 84 and 87-93 are indefinite because the claims appear to be missing at least an amplification step. The requirements set forth in claims 84 and 87-93 strongly suggest an amplification step, but neither these claims nor claim 67, from which they depend, recites an amplification step. The claims only explicitly recite a step of preparing a mixture and fail to

recite an amplification step or even incubation under conditions sufficient for amplification to occur. As a result, it is not clear whether the claims require an amplification step or not.

Claim 92 is further indefinite because the claim recites multiple trademarks/trade names (SYBR, TOTO, and PICOGREEN). Where a trademark or trade name is used in a claim as a limitation to identify or describe a particular material or product, the claim does not comply with the requirements of 35 U.S.C. 112(b) or 35 U.S.C. 112 (pre-AIA), second paragraph. See *Ex parte Simpson*, 218 USPQ 1020 (Bd. App. 1982). The claim scope is uncertain since the trademark or trade name cannot be used properly to identify any particular material or product. A trademark or trade name is used to identify a source of goods, and not the goods themselves. Thus, a trademark or trade name does not identify or describe the goods associated with the trademark or trade name. In the present case, the trademark/trade name is used to identify/describe particular types of fluorescent dyes and, accordingly, the identification/description is indefinite.

Claim 94 is indefinite because the claim appears to be missing at least an amplification step. The recitation in the claim for the method to be performed without an initial heat denaturation step suggests an amplification step, but the claim does not recite such a step. It only recites an active step of preparing a mixture. As a result, it is not clear whether the claim must include an amplification step or not.

Claim Rejections - 35 USC § 102

6. The following is a quotation of the appropriate paragraphs of pre-AIA 35 U.S.C. 102 that form the basis for the rejections under this section made in this Office action:

> A person shall be entitled to a patent unless –
>
> (b) the invention was patented or described in a printed publication in this or a foreign country or in public use or on sale in this country, more than one year prior to the date of application for patent in the United States.

Claims 67, 71-73, 76, 80, 83, 85, and 86 are rejected under pre-AIA 35 U.S.C. 102(b) as being anticipated by Van Ness et al. (WO 2003/008642 A1; IDS reference).

Regarding claims 67, 71-73, 76, 80, 83, 85, and 86, Van Ness teaches a method that comprises preparing a mixture comprising a target nucleic acid, polymerase, nicking enzyme, and two oligonucleotides, each of which has a nicking site and nicking enzyme binding site such that the nicking enzyme does not nick within the target nucleic acid (see, for example, page 10, line 14 - page 11, line 36, page 12, lines 5-23, and page 99, lines 1-13). The reference also teaches that the target nucleic acid may be a single-stranded viral RNA present in a blood, tissue, or biopsy sample obtained from a human (page 138, line 18 – page 139, line 1 and page 161, lines 4-30).

Claim Rejections - 35 USC § 103

7. The following is a quotation of pre-AIA 35 U.S.C. 103(a) which forms the basis for all obviousness rejections set forth in this Office action:

> (a) A patent may not be obtained though the invention is not identically disclosed or described as set forth in section 102 of this title, if the differences between the subject matter sought to be patented and the prior art are such that the subject matter as a whole would have been obvious at the time the invention was made to a person having ordinary skill in the art to which said subject matter pertains. Patentability shall not be negatived by the manner in which the invention was made.

This application currently names joint inventors. In considering patentability of the claims under pre-AIA 35 U.S.C. 103(a), the examiner presumes that the subject matter of the various claims was commonly owned at the time any inventions covered therein were made absent any evidence to the contrary. Applicant is advised of the obligation under 37 CFR 1.56 to

point out the inventor and invention dates of each claim that was not commonly owned at the time a later invention was made in order for the examiner to consider the applicability of pre-AIA 35 U.S.C. 103(c) and potential pre-AIA 35 U.S.C. 102(e), (f) or (g) prior art under pre-AIA 35 U.S.C. 103(a).

8. Claims 67-73, 76, 77, 80, and 83-94 are rejected under pre-AIA 35 U.S.C. 103(a) as being unpatentable over Wick et al. (US 6,063,604; cited on an IDS) in view of Kong et al. (US 6,191,267; cited on an IDS).

Regarding claims 67-73, 76, 77, 80, 83, and 94, Wick teaches a method that comprises subjecting a mixture of the following components to an amplification reaction: (i) a target nucleic acid, (ii) a polymerase, (iii) a restriction endonuclease capable of nicking at a hemi-modified recognition site, and (iv) two oligonucleotides, each comprising a recognition sequence for the restriction enzyme of (iii) (see, for example, Figure 1, column 10, line 39 – column 11, line 67, column 13, lines 7-52, and columns 25-28 (“Segregation and/or Amplification Scheme”); see also the Examples at columns 28-31). In the amplification method of Wick, the steps set forth in parts (a)-(f) of claim 68, parts (i) and (ii) of claim 69, and parts (a)-(c) of claim 70 occur (see Figures 1a-1b and the accompanying discussion at columns 25-28).The reference teaches that the target nucleic acid may be single-stranded viral RNA from an animal pathogen (see, for example, column 12, lines 17-21, 24-30, 34-38, and 62-66) and that samples containing the target nucleic acid may be isolated from tissue (e.g., liver, lung, kidney, and spleen), blood, tears, feces, urine, sputum, mucus, bone marrow, tissues or saliva samples obtained from humans

suspected of having been in contact with the virus (see, for example, column 12, lines 24-30 and 34-38 as well as column 13, lines 53-63).

Further regarding claim 94 and also regarding claim 84, Wick teaches that the method may be performed without an initial heat denaturation step (column 25, lines 28-33).

Regarding claim 86, Wick teaches that the nicking step does not result in nicking within the target polynucleotide sequence (see Figure 1b, for example).

Regarding claims 87 and 88, Wick teaches that the method is conducted isothermally at a temperatures within the claimed range (see, for example, column 13, lines 7-52, column 24, lines 33-67, and column 29, lines 2-8).

Regarding claims 91 and 92, Wick teaches detecting the amplification product using at least one of the required methods (see, for example, column 29, lines 11-44).

In the method of Wick, modified nucleotides (e.g., phosphorothioated nucleotides) are used to create a hemi-modified nucleic acid substrate for nicking (column 15, lines 17-53). As a result, the reference fails to teach the use of a nicking enzyme (e.g., Nt.BstNBI), as required by all of the claims. The reference also does not teach that the amplification time is about 1-20 minutes as required by claim 89. Further regarding claim 90, Wick also does not clearly teach that the amplification reaction is performed at a temperature that is higher than the melting temperature of the duplex formed between the first oligonucleotide and the target polynucleotide.

Kong describes methods for making and using Nt.BstNBI, which is a nicking enzyme (abstract and column 4, lines 21-50; see also Example 4 at column 14). The reference further teaches that the modified nucleotides typically used in strand displacement amplification are expensive and poorly incorporated by DNA polymerase, but that the use of a nicking enzyme

eliminates the need for their use (column 2, lines 4-45). Further regarding claim 89, Kong teaches that isothermal amplification reactions comprising strand displacement and use of a nicking enzyme may be completed in 20-60 minutes (column 14, lines 57-60).

It would have been *prima facie* obvious for one of ordinary skill in the art at the time of the invention to conduct the isothermal amplification method of Wick using the Nt.BstNBI nicking enzyme described by Kong. The ordinary artisan would have been motivated to do so in view of the teachings of Kong cited above, which indicate that using a nicking enzyme eliminates the need to use more expensive and poorly incorporated modified nucleotides in isothermal amplification reactions. The ordinary artisan would have had a reasonable expectation of success since Kong described methods for obtaining the enzyme (Examples 1-3 at columns 6-14) as well as a method for using it in an isothermal strand displacement amplification reaction (Example 4 at column 14).

Further regarding claim 89, it also would have been *prima facie* obvious for one of ordinary skill in the art at the time of the invention to conduct the amplification step in the method suggested by the teachings of Wick and Kong for a time within the claimed range of about 1-20 minutes. The ordinary artisan would have been motivated to do so with a reasonable expectation of success in view of the teachings of Kong in Example 4, which indicate that isothermal amplification reactions comprising strand displacement and use of a nicking enzyme may be completed in 20-60 minutes (column 14, lines 57-60).

Further regarding claim 90, it also would have been *prima facie* obvious for one of ordinary skill in the art at the time of the invention to conduct the amplification step in the method suggested by the teachings of Wick and Kong at a temperature that is higher than the

melting temperature of the duplex formed between the first oligonucleotide and the target polynucleotide. The ordinary artisan would have recognized that the temperature at which the amplification reaction is conducted is a results-effective variable and, accordingly, would have been motivated to conduct routine experimentation to determine an optimal temperature with a reasonable expectation of success. As discussed in MPEP 2144.05, performing routine experimentation to optimize results-effective variables is *prima facie* obvious in the absence of secondary considerations. In this case, no evidence of unexpected results has been presented.

Finally, further regarding claim 93, since the teachings of Wick and Kong suggest a method comprising all of the steps set forth in claim 67, it is inherent that the method results in at least 1E+9-fold amplification in about five minutes.

Thus, the methods of claims 67-73, 76, 77, 80, and 83-94 are *prima facie* obvious over Wick in view of Kong.

Double Patenting

9. The nonstatutory double patenting rejection is based on a judicially created doctrine grounded in public policy (a policy reflected in the statute) so as to prevent the unjustified or improper timewise extension of the "right to exclude" granted by a patent and to prevent possible harassment by multiple assignees. A nonstatutory double patenting rejection is appropriate where the claims at issue are not identical, but at least one examined application claim is not patentably distinct from the reference claim(s) because the examined application claim is either anticipated by, or would have been obvious over, the reference claim(s). See, e.g., *In re Berg*, 140 F.3d 1428, 46 USPQ2d 1226 (Fed. Cir. 1998); *In re Goodman*, 11 F.3d 1046, 29 USPQ2d

2010 (Fed. Cir. 1993); *In re Longi*, 759 F.2d 887, 225 USPQ 645 (Fed. Cir. 1985); *In re Van Ornum*, 686 F.2d 937, 214 USPQ 761 (CCPA 1982); *In re Vogel*, 422 F.2d 438, 164 USPQ 619 (CCPA 1970); and *In re Thorington*, 418 F.2d 528, 163 USPQ 644 (CCPA 1969).

A timely filed terminal disclaimer in compliance with 37 CFR 1.321(c) or 1.321(d) may be used to overcome an actual or provisional rejection based on a nonstatutory double patenting ground provided the reference application or patent either is shown to be commonly owned with this application, or claims an invention made as a result of activities undertaken within the scope of a joint research agreement. A terminal disclaimer must be signed in compliance with 37 CFR 1.321(b).

The USPTO internet Web site contains terminal disclaimer forms which may be used. Please visit http://www.uspto.gov/forms/. The filing date of the application will determine what form should be used. A web-based eTerminal Disclaimer may be filled out completely online using web-screens. An eTerminal Disclaimer that meets all requirements is auto-processed and approved immediately upon submission. For more information about eTerminal Disclaimers, refer to http://www.uspto.gov/patents/process/file/efs/guidance/eTD-info-I.jsp.

10. Claims 67-73, 76, 77, 80, and 83-94 are provisionally rejected on the ground of nonstatutory double patenting as being unpatentable over claims 67-69, 74-76, 79, 80, 83, and 85-96 of copending Application No. 14/067,623. Although the claims at issue are not identical, they are not patentably distinct from each other because the claims of the '623 application overlap in scope with the claimed methods and recite all of their limitations.

This is a provisional nonstatutory double patenting rejection because the patentably indistinct claims have not in fact been patented.

11. Claims 67-73, 76, 77, 80, and 83-94 are provisionally rejected on the ground of nonstatutory double patenting as being unpatentable over claims 1-9, 12, 14-17, 19-42, and 44-47 of copending Application No. 12/173,020. Although the claims at issue are not identical, they are not patentably distinct from each other because the claims of the ‘020 application are also drawn to an isothermal amplification method comprising the use of nicking enzymes and primers having nicking enzyme recognition and cleavage sites (see, in particular, claims 1, 41, 42, 44, and 47). The claims of the '020 application also suggest the use of single-stranded viral RNA in a sample from an animal as the target polynucleotide (see, in particular, claims 9, 12, 14, 45, and 46). Further regarding the instant claim 77, mucus, saliva, and sputum were generally known in the art to be useful samples for nucleic acid amplification procedures. Accordingly, this claim is also not patentably distinct from the claims of the '020 application. Further regarding the instant claim 90, although the claims of the '020 application do not state that the amplification step is performed at a temperature that is higher than the melting temperature of the duplex formed between the first oligonucleotide template and the target polynucleotide, the ordinary artisan would have recognized that the temperature at which the amplification reaction is conducted is a results-effective variable and, accordingly, would have been motivated to conduct routine experimentation to determine an optimal temperature with a reasonable expectation of success. As discussed in MPEP 2144.05, performing routine experimentation to optimize results-effective variables is *prima facie* obvious in the absence of secondary considerations. In this case, no

evidence of unexpected results has been presented. Thus, the instant claims 67-73, 76, 77, 80, and 83-94 are not patentably distinct from claims 1-9, 12, 14-17, 19-42, and 44-47 of the ‘020 application.

This is a provisional nonstatutory double patenting rejection because the patentably indistinct claims have not in fact been patented.

12. Claims 67-73, 76, 77, 80, 83, 84, 86-88, and 90-94 are provisionally rejected on the ground of nonstatutory double patenting as being unpatentable over claims 125-130 of copending Application No. 11/778,018 in view of Wick et al. (US 6,063,604; cited on an IDS).

The instant claims are drawn to method for isothermal amplification of a single-stranded viral RNA target that comprises the use of nicking enzymes. Claims 125-130 of the ‘018 application are also drawn to a method for isothermal amplification of a single-stranded RNA target that comprises the use of nicking enzymes. The claims of the ‘018 application disclose or suggest all of the limitations of the instant claims 67-73, 76, 77, 80, 83, 84, 86-88, and 90-94, but they do not state that the RNA target is a single-stranded viral RNA contained in a sample obtained from an animal as required by the instant claims. The claims of the '018 application also do not state that an initial heat denaturation step is omitted as required by the instant claims 84 and 94 or that the method further comprises detection of the amplification product as required by the instant claims 91 and 92. Further, the claims of the ‘018 application do not teach the requirements of the instant claim 90.

Wick teaches a method that comprises subjecting a mixture of the following components to an iosothermal amplification reaction: (i) a target nucleic acid, (ii) a polymerase, (iii) a

restriction endonuclease capable of nicking at a hemi-modified recognition site, and (iv) two oligonucleotides, each comprising a recognition sequence for the restriction enzyme of (iii) (see, for example, Figure 1, column 10, line 39 – column 11, line 67, column 13, lines 7-52, and columns 25-28 ("Segregation and/or Amplification Scheme"); see also the Examples at columns 28-31). The reference teaches that the target nucleic acid may be single-stranded viral RNA from an animal pathogen (see, for example, column 12, lines 17-21, 24-30, 34-38, and 62-66) and that samples containing the target nucleic acid may be isolated from tissue (e.g., liver, lung, kidney, and spleen), blood, tears, feces, urine, sputum, mucus, bone marrow, tissues or saliva samples obtained from humans suspected of having been in contact with the virus (see, for example, column 12, lines 24-30 and 34-38 as well as column 13, lines 53-63).

Further regarding claim 94 and also regarding claim 84, Wick teaches that the method may be performed without an initial heat denaturation step (column 25, lines 28-33).

Regarding claims 91 and 92, Wick teaches detecting the amplification product using at least one of the required methods (see, for example, column 29, lines 11-44).

It would have been *prima facie* obvious for one of ordinary skill in the art at the time of the invention to conduct the practice the isothermal amplification method recited in the claims of the '018 application using a single-stranded viral RNA contained in a sample from an animal as the target nucleic acid. The ordinary artisan would have been motivated to do so with a reasonable expectation of success in view of the teachings of Wick, which indicate that such samples and targets may be used in a similar isothermal amplification method comprising nicking and strand displacement. The ordinary artisan also would have been motivated to conduct the method without an initial heat denaturation step since Wick taught that heat

denaturation could be substituted with pH denaturation (column 25, lines 28-33). The ordinary artisan also would have been motivated to detect the amplification product using a method described by Wick since detection of amplification products was routine and conventional in the art at the time of the invention. Finally, further regarding the instant claim 90, it would have been *prima facie* obvious for one of ordinary skill in the art at the time of the invention to conduct the amplification step at a temperature that is higher than the melting temperature of the duplex formed between the first oligonucleotide and the target polynucleotide. The ordinary artisan would have recognized that the temperature at which the amplification reaction is conducted is a results-effective variable and, accordingly, would have been motivated to conduct routine experimentation to determine an optimal temperature with a reasonable expectation of success. As discussed in MPEP 2144.05, performing routine experimentation to optimize results-effective variables is *prima facie* obvious in the absence of secondary considerations. In this case, no evidence of unexpected results has been presented.

Thus, the instant claims 67-73, 76, 77, 80, 83, 84, 86-88, and 90-94 are not patentably distinct from claims 125-130 of the '018 application in view of Wick.

This is a provisional nonstatutory double patenting rejection because the patentably indistinct claims have not in fact been patented.

13. Claims 85 and 89 are provisionally rejected on the ground of nonstatutory double patenting as being unpatentable over claims 125-130 of copending Application No. 11/778,018 in view of Wick et al. (US 6,063,604; cited on an IDS) and further in view of Kong et al. (US 6,191,267; cited on an IDS)

As discussed above, the instant claims 67-73, 76, 77, 80, 83, 84, 86-88, and 90-94 are not patentably distinct from claims 125-130 of the ‘018 application in view of Wick.

Neither Wick nor the claims of the ‘018 application teach conducting the amplification reaction for a time within the range set forth in claim 89 or the use of Nt.BstNBI as the nicking enzyme as required by the instant claim 85.

Kong describes methods for making and using Nt.BstNBI, which is a nicking enzyme suitable for use in isothermal strand displacement amplification methods comprising a nicking step (abstract and column 4, lines 21-50; see also Example 4 at column 14). The reference also teaches that isothermal amplification reactions comprising strand displacement and use of a nicking enzyme may be completed in 20-60 minutes (column 14, lines 57-60).

It would have been *prima facie* obvious for the ordinary artisan practicing the method suggested by the claims of the '018 application and Wick to select Nt.BstNBI as the nicking enzyme. The ordinary artisan would have been motivated to do so with a reasonable expectation of success since Kong taught that this nicking enzyme was suitable for use in isothermal strand displacement amplification reactions comprising a nicking step and also eliminated the need to use expensive and poorly incorporated modified nucleotides. It also would have been *prima facie* obvious for the ordinary artisan practicing the method suggested by the claims of the '018 application and Wick to conduct the amplification step for a time within the claimed range of about 1-20 minutes. The ordinary artisan would have been motivated to do so with a reasonable expectation of success in view of the teachings of Kong in Example 4, which indicate that isothermal amplification reactions comprising strand displacement and use of a nicking enzyme may be completed in 20-60 minutes (column 14, lines 57-60).

Thus, the instant claims 85 and 89 are not patentably distinct from claims 125-130 of the '018 application in view of Wick and further in view of Kong.

This is a provisional nonstatutory double patenting rejection because the patentably indistinct claims have not in fact been patented.

Conclusion

14. No claims are currently allowable.

Any inquiry concerning this communication or earlier communications from the examiner should be directed to Angela M. Bertagna whose telephone number is (571)272-8291. The examiner can normally be reached on Monday-Friday, 9-5.

If attempts to reach the examiner by telephone are unsuccessful, the examiner's supervisor, Gary Benzion can be reached on (571)272-0782. The fax phone number for the organization where this application or proceeding is assigned is 571-273-8300.

Information regarding the status of an application may be obtained from the Patent Application Information Retrieval (PAIR) system. Status information for published applications may be obtained from either Private PAIR or Public PAIR. Status information for unpublished applications is available through Private PAIR only. For more information about the PAIR system, see http://pair-direct.uspto.gov. Should you have questions on access to the Private PAIR system, contact the Electronic Business Center (EBC) at 866-217-9197 (toll-free). If you would like assistance from a USPTO Customer Service Representative or access to the automated information system, call 800-786-9199 (IN USA OR CANADA) or 571-272-1000.

/Angela M. Bertagna/
Primary Examiner, Art Unit 1637

EAST Search History (Prior Art)

Ref #	Hits	Search Query	DBs	Default Operator	Plurals	Time Stamp
S1	9	(ionian).as. and (nicking or nick or nicked or nickase)	US-PGPUB; USPAT; FPRS; EPO; JPO; DERWENT	OR	OFF	2014/10/10 09:30
S2	3	(ionian).as. and (nicking or nick or nicked or nickase).clm.	US-PGPUB; USPAT; FPRS; EPO; JPO; DERWENT	OR	OFF	2014/10/10 09:32
S3	7	(van adj1 ness).in. and (nicking or nick or nicked or nickase).clm.	US-PGPUB; USPAT; FPRS; EPO; JPO; DERWENT	OR	OFF	2014/10/10 09:32
S4	7	S3 not S1	US-PGPUB; USPAT; FPRS; EPO; JPO; DERWENT	OR	OFF	2014/10/10 09:32
S5	7	(galas).in. and (nicking or nick or nicked or nickase).clm.	US-PGPUB; USPAT; FPRS; EPO; JPO; DERWENT	OR	OFF	2014/10/10 09:35
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EAST Search History (Prior Art)

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S27	11	(ribonucleic or rna) near8 nicking same (amplif$) same (isothermal$ or (constant adj1 temperature))	US-PGPUB; USPAT; FPRS; EPO; JPO; DERWENT	OR	OFF	2014/10/14 09:59
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S30	48	S29 not S28 not S23	US-PGPUB; USPAT; FPRS; EPO; JPO; DERWENT	OR	OFF	2014/10/14 09:59
S31	3	wo-03008642-$.did.	US-PGPUB; USPAT; FPRS; EPO; JPO; DERWENT	OR	OFF	2014/10/14 12:01
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S34	10	(van adj1 ness).in. and (amplif$ same minute$)	US-PGPUB; USPAT	OR	OFF	2014/10/14 12:48

EAST Search History (Prior Art)

S35	63	S33 not "l1"	US-PGPUB; USPAT	OR	OFF	2014/10/14 12:49
S36	61	S33 not S34	US-PGPUB; USPAT	OR	OFF	2014/10/14 12:49
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S41	523	(sda or (strand adj1 displacement adj1 amplification)) near8 rna	US-PGPUB; USPAT; FPRS; EPO; JPO; DERWENT	OR	OFF	2014/10/14 18:43

EAST Search History (Interference)

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Substitute Disclosure Form U.S. Department of Commerce Patent and Trademark Office	Attorney Docket No. 30171-0025002	Application No. 14/067,620
Information Disclosure Statement by Applicant (Use several sheets if necessary)	First Named Inventor Brian K. Maples	
(37 CFR §1.98(b))	Filing Date October 30, 2013	Group Art Unit 1637

U.S. Patent Documents

Examiner Initial	Desig. ID	Document Number	Publication Date	Patentee	Class	Subclass	Filing Date If Appropriate
	1						

Foreign Patent Documents or Published Foreign Patent Applications

Examiner Initial	Desig. ID	Document Number	Publication Date	Country or Patent Office	Class	Subclass	Translation Yes	Translation No
/A.B./	2	EP2660333	11/06/2013	Europe				
/A.B./	3	EP2657350	10/30/2013	Europe				
/A.B./	4	WO2007/028833	03/15/2007	WIPO				
/A.B./	5	WO94/03635	02/17/1997	WIPO				
	6							

Other Documents (include Author, Title, Date, and Place of Publication)

Examiner Initial	Desig. ID	Document
/A.B./	7	Sequence of vector pUC19, downloaded from http://genome-www.stanford.edu/vectordb/vector_descrip/COMPLET... On March 27, 2014
/A.B./	8	Notice of Opposition in corresponding EP Application No. 08781827.4, dated May 6, 2014, pages 1-36
	9	

Examiner Signature /Angela Bertagna/	Date Considered 10/10/2014
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Substitute Disclosure Form

Substitute Disclosure Form U.S. Department of Commerce Patent and Trademark Office	Attorney Docket No. 30171-0025002	Application No. 14/067,620
Information Disclosure Statement by Applicant (Use several sheets if necessary) (37 CFR §1.98(b))	First Named Inventor Brian K. Maples	
	Filing Date October 30, 2013	Group Art Unit 1637

U.S. Patent Documents

Examiner Initial	Desig. ID	Document Number	Publication Date	Patentee	Class	Subclass	Filing Date If Appropriate
	1						

Foreign Patent Documents or Published Foreign Patent Applications

Examiner Initial	Desig. ID	Document Number	Publication Date	Country or Patent Office	Class	Subclass	Translation	
							Yes	No
	2							

Other Documents (include Author, Title, Date, and Place of Publication)

Examiner Initial	Desig. ID	Document
/A.B./	3	Copy of English translation of Chinese Third Office action, for corresponding Chinese application CN 200880105424.7. dated February 8, 2014
/A.B./	4	Notification of the Final Rejection for corresponding Chinese application CN 200880105424.7. dated June 5, 2014
	5	
	6	

Examiner Signature /Angela Bertagna/	Date Considered 10/10/2014
EXAMINER: Initials citation considered. Draw line through citation if not in conformance and not considered. Include copy of this form with next communication to applicant.	

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BIB DATA SHEET

CONFIRMATION NO. 4288

SERIAL NUMBER	FILING or 371(c) DATE	CLASS	GROUP ART UNIT	ATTORNEY DOCKET NO.
14/067,620	10/30/2013 RULE	435	1637	30171-0025002 / ITI-001

APPLICANTS
Ionian Technologies, Inc., San Diego, CA, Assignee (with 37 CFR 1.172 Interest);

INVENTORS
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Andrew P. Miller, San Diego, CA;
Jarrod Provins, Dana Point, CA;
Richard Roth, Carlsbad, CA;
Jeffrey Mandell, San Diego, CA;

** **CONTINUING DATA** *************************
This application is a CON of 11/778,018 07/14/2007

** **FOREIGN APPLICATIONS** *************************

** **IF REQUIRED, FOREIGN FILING LICENSE GRANTED** **
11/21/2013

Foreign Priority claimed ☐ Yes ☑ No 35 USC 119(a-d) conditions met ☐ Yes ☑ No Verified and Acknowledged /ANGELA MARIE BERTAGNA/ Examiner's Signature	☐ Met after Allowance Initials	STATE OR COUNTRY	SHEETS DRAWINGS	TOTAL CLAIMS	INDEPENDENT CLAIMS
		CA	24	29	3

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UNITED STATES

TITLE

Nicking and Extension Amplification Reaction for the Exponential Amplification of Nucleic Acids

FILING FEE RECEIVED	FEES: Authority has been given in Paper	
2620	FEES: Authority has been given in Paper No.________ to charge/credit DEPOSIT ACCOUNT No.________ for following:	☐ All Fees ☐ 1.16 Fees (Filing) ☐ 1.17 Fees (Processing Ext. of time) ☐ 1.18 Fees (Issue) ☐ Other ________ ☐ Credit

BIB (Rev. 05/07).

Substitute Disclosure Form U.S. Department of Commerce Patent and Trademark Office	Attorney Docket No. 30171-0025002	Application No. Not Yet Assigned
Information Disclosure Statement by Applicant (Use several sheets if necessary) (37 CFR §1.98(b))	Applicant Ionian Technologies Inc.	
	Filing Date Herewith	Group Art Unit Unknown

U.S. Patent Documents

Examiner Initial	Desig. ID	Document Number	Publication Date	Patentee	Class	Subclass	Filing Date If Appropriate
/A.B./	1	5,210,015	5/11/1993	Gelfand et al.			
	2	5,270,184	12/14/1993	Walker et al.			
	3	5,397,698	3/19/1995	Goodman et al.			
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	5	5,747,246	5/5/1998	Pannetier et al.			
	6	5,747,255	5/5/1998	Brenner			
	7	5,804,375	9/8/1998	Gelfand et al.			
	8	5,846,717	12/8/1998	Brow et al.			
	9	5,985,557	11/16/1999	Prudent et al.			
	10	6,063,604	05/16/2000	Wick et al.			
	11	6,087,133	07/11/2000	Dattagupta et al.			
	12	6,090,552	07/18/2000	Nazarenko et al.			
	13	6,110,677	08/29/2000	Western et al.			
	14	6,130,038	10/10/2000	Becker et al.			
	15	6,191,267	02/20/2001	Kong et al.			
	16	6,214,587	04/10/2001	Dattagupta et al.			
	17	6,251,600	06/26/2001	Winger et al.			
	18	6,261,768	07/17/2001	Todd et al.			
	19	6,316,200	11/13/2001	Nadeau et al.			
	20	6,348,314	02/19/2002	Prudent et al.			
	21	6,350,580	02/26/2002	Sorge			
	22	6,632,611	10/14/2003	Su et al.			
	23	6,656,680	12/02/2003	Nadeau et al.			
	24	6,692,917	02/17/2004	Neri et al.			
	25	6,743,582	06/01/2004	Nadea et al.			
	26	6,861,222	03/01/2005	Ward et al.			
/A.B./	27	6,884,586	04/26/2005	Van Ness et al.			

Examiner Signature /Angela Bertagna/	Date Considered 10/10/2014
EXAMINER: Initials citation considered. Draw line through citation if not in conformance and not considered. Include copy of this form with next communication to applicant.	

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Substitute Disclosure Form U.S. Department of Commerce Patent and Trademark Office	Attorney Docket No. 30171-0025002	Application No. Not Yet Assigned
Information Disclosure Statement by Applicant (Use several sheets if necessary)	Applicant Ionian Technologies Inc.	
(37 CFR §1.98(b))	Filing Date Herewith	Group Art Unit Unknown

U.S. Patent Documents

Examiner Initial	Desig. ID	Document Number	Publication Date	Patentee	Class	Subclass	Filing Date If Appropriate
/A.B./	28	6,893,819	05/17/2005	Sorge			
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/A.B./	36	WO 00/28084	5/18/2000	WIPO				
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/A.B./	38	WO 04/067726	8/12/2004	WIPO				
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/A.B./	46	Saiki et al., "Primer-Directed Enzymatic Amplification of DNA with a Thermostable DNA Polymerase," Science, 239:487-491, 1988

Examiner Signature /Angela Bertagna/	Date Considered 10/10/2014
EXAMINER: Initials citation considered. Draw line through citation if not in conformance and not considered. Include copy of this form with next communication to applicant.	

Substitute Disclosure Form

Substitute Disclosure Form **Information Disclosure Statement by Applicant** (Use several sheets if necessary) (37 CFR §1.98(b))	U.S. Department of Commerce Patent and Trademark Office	Attorney Docket No. 30171-0025002	Application No. Not Yet Assigned
		Applicant Ionian Technologies Inc.	
		Filing Date Herewith	Group Art Unit Unknown

Other Documents (include Author, Title, Date, and Place of Publication)

Examiner Initial	Desig. ID	Document
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/A.B./	48	Office Action in U.S. Application No. 12/173,020 , mailed December 27, 2010
/A.B./	49	Restriction Requirement in U.S. Application No. 12/173,020 , mailed September 17, 2010
/A.B./	50	Copy of the International Search Report, for the corresponding PCT Application No. PCT/US2008/070023, dated January 19, 2009.
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/A.B./	53	Copy of English translation and Chinese Office action, for corresponding Chinese application CN 200880105424.7. dated July 23, 2012
/A.B./	54	Copy of AU Office Action for corresponding AU Application No. 2008276118, 4 pages, dated May 1, 2013
/A.B./	55	Copy of English translation of Chinese Second Office action, for corresponding Chinese application CN 200880105424.7. dated June 5, 2013
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Examiner Signature /Angela Bertagna/	Date Considered 10/10/2014
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Substitute Disclosure Form

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US Patent & Trademark Office: U.S. DEPARTMENT OF COMMERCE
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Substitute for form 1449A/PTO **INFORMATION DISCLOSURE STATEMENT BY APPLICANT** *(Use as many sheets as necessary)*	*Complete if Known*	
	Application Number	11/778,018
	Filing Date	July 14, 2007
	First Named Inventor	MAPLES, Brian K.
	Group Art Unit	1635
	Examiner Name	NOT YET ASSIGNED
Sheet 1 of 2	Attorney Docket No: ITI-1001-UT	

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Examiner Initials *	Cite No.[1]	USP Document Number	Publication Date	Name of Patentee or Applicant of cited Document	Pages,Columns,Lines,Where Relevant Passages or Relevant Figures Appear
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EXAMINER /Angela Bertagna/ **DATE CONSIDERED** 10/10/2014

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INFORMATION DISCLOSURE STATEMENT BY APPLICANT

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First Named Inventor	MAPLES, Brian K.
Group Art Unit	1635
Examiner Name	NOT YET ASSIGNED

Attorney Docket No: ITI-1001-UT

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Examiner Initials*	Cite No.[1]	Foreign Document No	Publication Date	Name of Patentee or Applicant of cited Document	Pages,Columns,Lines, Where Relevant Passages or Relevant Figures Appear	T[2]
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Examiner Initials*	Cite No[1]	Include name of the author (in CAPITAL LETTERS), title of the article (when appropriate), title of the item (book, magazine, journal, serial, symposium, catalog, etc.), date, page(s), volume-issue number(s), publisher, city and/or country where published.	T[2]

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INFORMATION DISCLOSURE STATEMENT BY APPLICANT

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Sheet 1 of 3

Complete if Known	
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Sheet 2 of 3

Complete if Known	
Application Number	11/778,018
Filing Date	July 14, 2007
First Named Inventor	MAPLES, Brian K.
Group Art Unit	1635
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Attorney Docket No: ITI-1001-UT	

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	Filing Date	July 14, 2007
	First Named Inventor	MAPLES, Brian K.
	Group Art Unit	1635
	Examiner Name	NOT YET ASSIGNED
Sheet 3 of 3	Attorney Docket No: ITI-1001-UT	

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/A.B./	**A49.**	Reinhart et al., 2002, Gene & Dev., 16, 1616-1626	
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/A.B./	**A55.**	Volpe et al., 2002, Science, 297, 1833-1837	
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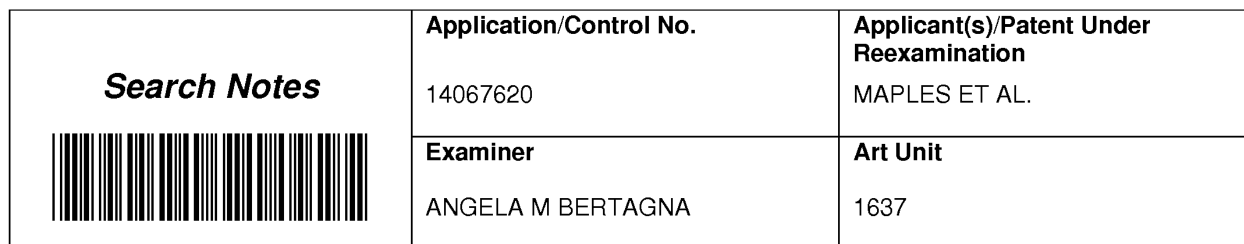
Search Notes 	Application/Control No. 14067620	Applicant(s)/Patent Under Reexamination MAPLES ET AL.
	Examiner ANGELA M BERTAGNA	**Art Unit** 1637

CPC- SEARCHED

Symbol	Date	Examiner

CPC COMBINATION SETS - SEARCHED

Symbol	Date	Examiner

US CLASSIFICATION SEARCHED

Class	Subclass	Date	Examiner

SEARCH NOTES

Search Notes	Date	Examiner
searched all inventors by name	10/10/2014	amb
EAST search history attached	10/10/2014; 10/14/2014	amb
Google Scholar (search terms included "nicking", "isothermal", and "RNA")	10/10/2014; 10/14/2014	amb
reviewed related cases - 14067623 & 11778018	10/10/2014	amb

INTERFERENCE SEARCH

US Class/ CPC Symbol	US Subclass / CPC Group	Date	Examiner

NICKING AND EXTENSION AMPLIFICATION REACTION FOR THE EXPONENTIAL AMPLIFICATION OF NUCLEIC ACIDS

Related Applications

This application is a continuation of U.S. Application Serial No. 11/778,018, filed July 14, 2007, the entire contents of which are hereby incorporated.

Field of the Invention

The invention is in general directed to the rapid exponential amplification of short DNA or RNA sequences at a constant temperature.

Background

The field of *in vitro* diagnostics is quickly expanding as the need for systems that can rapidly detect the presence of harmful species or determine the genetic sequence of a region of interest is increasing exponentially. Current molecular diagnostics focus on the detection of biomarkers and include small molecule detection, immuno-based assays, and nucleic acid tests. The built-in specificity between two complementary nucleic acid strands allows for fast and specific recognition using unique DNA or RNA sequences, the simplicity of which makes a nucleic acid test an attractive prospect. Identification of bacterial and viral threat agents, genetically modified food products, and single nucleotide polymorphisms for disease management are only a few areas where the advancement of these molecular diagnostic tools becomes extremely advantageous. To meet these growing needs, nucleic acid amplification technologies have been developed and tailored to these needs of specificity and sensitivity.

Historically, the most common amplification technique is the polymerase chain reaction (PCR), which has in many cases become the gold standard for detection methods because of its reliability and specificity. This technique requires the cycling of temperatures to proceed through the steps of denaturation of the dsDNA, annealing of short oligonucleotide primers, and extension of the primer along the template by a thermostable polymerase. Though many new advances in engineering have successfully shortened these reaction times to 20-30 minutes, there is still a steep power requirement to meet the needs of these thermocycling units.

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

First Named Inventor	:	Brian K. Maples	Art Unit	:	1637
Serial No.	:	14/067,620	Examiner	:	Olayinka A. Oyeyemi
Filed	:	October 30, 2013	Conf. No.	:	4288
Title	:	NICKING AND EXTENSION AMPLIFICATION REACTION FOR THE EXPONENTIAL AMPLIFICATION OF NUCLEIC ACIDS			

Commissioner for Patents
P.O. Box 1450
Alexandria, VA 22313-1450

PRELIMINARY AMENDMENT

Prior to examination, please amend the application as indicated on the following pages.

Amendments to the Claims:

This listing of claims replaces all prior versions and listings of claims in the application:

Listing of Claims:

1. - 66. (Canceled)

67. (Previously Presented) A method, comprising:

preparing a mixture comprising:

(i) a target nucleic acid present in a sample obtained from an animal, the target nucleic acid having a target polynucleotide sequence,

(ii) a polymerase,

(iii) a nicking enzyme,

(iv) a first oligonucleotide comprising a nicking site and a nicking enzyme binding site, and

(v) a second oligonucleotide comprising a nicking site and a nicking enzyme binding site.

68. (Previously Presented) The method of claim 67, wherein the target polynucleotide sequence is amplified from steps comprising:

(a) forming a first duplex comprising the target polynucleotide sequence and the first oligonucleotide;

(b) extending, using the polymerase, the first oligonucleotide along the target polynucleotide sequence to form an extended first oligonucleotide comprising a sequence complementary to the second oligonucleotide;

(c) forming a second duplex comprising the second oligonucleotide and the extended first oligonucleotide;

(d) extending, using the polymerase, the second oligonucleotide along the extended first oligonucleotide to form a third duplex comprising an extended second oligonucleotide

comprising a sequence complementary to the first oligonucleotide and a first double-stranded nicking enzyme binding site;

(e) nicking, with the nicking enzyme, the first nicking site on the third duplex to produce a fourth duplex comprising the extended second oligonucleotide and a fragment of the extended first oligonucleotide; and

(f) extending, using the polymerase, the fragment of the extended first oligonucleotide along the extended second oligonucleotide of the fourth duplex to produce a double-stranded nucleic acid product and a second double-stranded nicking enzyme binding site.

69. (Previously Presented) The method of claim 68, wherein the double-stranded nucleic acid product comprises:

i) a first strand and a second strand, wherein the first strand comprises a first polynucleotide sequence corresponding to the target polynucleotide sequence and the second strand comprises a second polynucleotide sequence complementary to the target polynucleotide sequence, and

ii) first and second double-stranded nicking sites spaced apart by the target polynucleotide sequence.

70. (Previously Presented) The method of claim 68, further comprising the steps of:

a) nicking, using the nicking enzyme, the first nicking site of the double-stranded nucleic acid product to produce a fifth duplex comprising a first polynucleotide sequence corresponding to the target polynucleotide sequence and a fragment of the first oligonucleotide, and nicking, using the nicking enzyme, the second nicking site of the double-stranded nucleic acid product to produce a sixth duplex comprising a second polynucleotide sequence complementary to the target polynucleotide sequence and a fragment of the second oligonucleotide;

b) extending, using the polymerase, the fragment of the first oligonucleotide along the first polynucleotide sequence of the fifth duplex to produce a first double stranded product comprising a copy of the nicking site and a copy of the first polynucleotide sequence and extending, using the polymerase, the fragment of the second oligonucleotide along the second

polynucleotide sequence of the sixth duplex to produce a second double stranded product comprising a copy of the nicking site and a copy of the second polynucleotide sequence; and

c) nicking, using the nicking enzyme, the copy of the nicking site of the first double stranded product to release a copy of the first polynucleotide sequence and nicking, using the nicking enzyme, the copy of the nicking site of the second double stranded product to release a copy of the second polynucleotide sequence.

71. (Previously Presented) The method of claim 67, wherein the animal is a human.

72. (Previously Presented) The method of claim 67, wherein the target nucleic acid is obtained from an animal pathogen.

73. (Previously Presented) The method of claim 72, wherein the animal pathogen is a single-stranded DNA virus, double-stranded DNA virus, or single-stranded RNA virus.

74. (Withdrawn) The method of claim 72, wherein the animal pathogen is a bacterium.

75. (Withdrawn) The method of claim 72, wherein the animal pathogen contains spores and the target polynucleotide is amplified from the spores without the need for lysis of the spores.

76. (Previously Presented) The method of claim 67, wherein the sample obtained from an animal is obtained from the blood, bone marrow, mucus, lymph, hard tissues (e.g. liver, spleen, kidney, lung or ovary), biopsies, sputum, saliva, tears, faeces or urine of the animal.

77. (Canceled)

78. (Withdrawn) The method of claim 67, wherein the target nucleic acid is double-stranded DNA.

79. (Withdrawn) The method of claim 67, wherein the target nucleic acid is single-stranded DNA.

80. (Previously Presented) The method of claim 67, wherein the target nucleic acid is RNA.

81. (Withdrawn) The method of claim 67, wherein the target nucleic acid is selected from the group consisting of genomic DNA, plasmid DNA, viral DNA, mitochondrial DNA, cDNA, synthetic double-stranded DNA and synthetic single-stranded DNA.

82. (Withdrawn) The method of claim 81, wherein the target nucleic acid is genomic DNA.

83. (Previously Presented) The method of claim 67, wherein the target nucleic acid is viral DNA or viral RNA.

84. (Previously Presented) The method of claim 67, which is performed without an initial heat denaturation step.

85. – 86. (Canceled)

87. (Previously Presented) The method of claim 67, which is performed without the use of temperature cycling.

88. (Previously Presented) The method of claim 67, which is performed at about 55°C-59°C.

89. (Previously Presented) The method of claim 67, which is performed at a constant temperature for about 1 to 20 minutes.

90. (Previously Presented) The method of claim 68, which is performed at a temperature higher than the melting temperature of the first oligonucleotide/target polynucleotide sequence complex.

91. (Previously Presented) The method of claim 67, further comprising detecting amplification product.

92. (Canceled)

93. (Previously Presented) The method of claim 67, wherein the target polynucleotide sequence is amplified 1E+9-fold or more in about five minutes.

94. (Previously Presented) A method, comprising:

preparing a mixture comprising:

(i) a target nucleic acid present in a sample obtained from an animal, the target nucleic acid having a target polynucleotide sequence,

(ii) a polymerase,

(iii) a nicking enzyme,

(iv) a first oligonucleotide comprising a nicking site and a nicking enzyme binding site, and

(v) a second oligonucleotide comprising a nicking site and a nicking enzyme binding site;

which method is performed without an initial heat denaturation step.

95. (Previously Presented) A method, comprising:

preparing a mixture comprising:

(i) genomic DNA present in a sample obtained from an animal, the genomic DNA having a target polynucleotide sequence,

(ii) a polymerase,

(iii) a nicking enzyme,

(iv) a first oligonucleotide comprising a nicking site and a nicking enzyme binding site, and

(v) a second oligonucleotide comprising a nicking site and a nicking enzyme binding site; which method is performed without an initial heat denaturation step.

96. (New) The method of claim 67, wherein the target nucleic acid present in the sample has not been purified.

REMARKS

Applicant respectfully request entry of the amendments submitted herein. Upon entry of the present amendment, claims 67-73, 76, 80, 83-84, 87-91 and 93-96 will be pending. Claims 1-67 were previously canceled, and claims 77, 85-86 and 92 are newly canceled. Claims 74-75, 78-79 and 81-82 have been withdrawn, and claim 96 has been added.

Applicant asks that all claims be examined in view of the amendment to the claims.

No fee is believed to be due, however, please apply any other necessary charges or credits to Deposit Account 06-1050, referencing the above attorney docket number.

Respectfully submitted,

Date: October 15, 2014______________

/Ian J.S. Lodovice, Reg. No. 59,749/__________
Ian J. Lodovice
Reg. No. 59,749

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23304755.doc

Electronic Acknowledgement Receipt

EFS ID:	20426620
Application Number:	14067620
International Application Number:	
Confirmation Number:	4288
Title of Invention:	Nicking and Extension Amplification Reaction for the Exponential Amplification of Nucleic Acids
First Named Inventor/Applicant Name:	Brian K. Maples
Customer Number:	26161
Filer:	Ian J.S. Lodovice/Mary Florczak
Filer Authorized By:	Ian J.S. Lodovice
Attorney Docket Number:	30171-0025002 / ITI-001
Receipt Date:	15-OCT-2014
Filing Date:	30-OCT-2013
Time Stamp:	17:30:19
Application Type:	Utility under 35 USC 111(a)

Payment information:

Submitted with Payment	no

File Listing:

Document Number	Document Description	File Name	File Size(Bytes)/ Message Digest	Multi Part /.zip	Pages (if appl.)
1	First Action Interview - Schedule Interview request	30171Request.pdf	138204 cd466b556ab85a1af7167e1246f9c000709ca55e	no	1

Warnings:

Information:

2		30171Prelim.pdf	82475 4bb4b11ac85172486531fcc308d710fa6b860373	yes	8

	Multipart Description/PDF files in .zip description		
	Document Description	**Start**	**End**
	Preliminary Amendment	1	1
	Claims	2	7
	Applicant Arguments/Remarks Made in an Amendment	8	8

Warnings:

Information:

Total Files Size (in bytes):	220679

This Acknowledgement Receipt evidences receipt on the noted date by the USPTO of the indicated documents, characterized by the applicant, and including page counts, where applicable. It serves as evidence of receipt similar to a Post Card, as described in MPEP 503.

New Applications Under 35 U.S.C. 111
If a new application is being filed and the application includes the necessary components for a filing date (see 37 CFR 1.53(b)-(d) and MPEP 506), a Filing Receipt (37 CFR 1.54) will be issued in due course and the date shown on this Acknowledgement Receipt will establish the filing date of the application.

National Stage of an International Application under 35 U.S.C. 371
If a timely submission to enter the national stage of an international application is compliant with the conditions of 35 U.S.C. 371 and other applicable requirements a Form PCT/DO/EO/903 indicating acceptance of the application as a national stage submission under 35 U.S.C. 371 will be issued in addition to the Filing Receipt, in due course.

New International Application Filed with the USPTO as a Receiving Office
If a new international application is being filed and the international application includes the necessary components for an international filing date (see PCT Article 11 and MPEP 1810), a Notification of the International Application Number and of the International Filing Date (Form PCT/RO/105) will be issued in due course, subject to prescriptions concerning national security, and the date shown on this Acknowledgement Receipt will establish the international filing date of the application.

PTO/SB/413C (10-09)
Approved for use through 07/31/2012. OMB 0651-0031
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REQUEST FOR FIRST-ACTION INTERVIEW (PILOT PROGRAM)

Attorney Docket Number: 30171-0025002	Application Number (if known): 14/067,620	Filing date: October 30, 2013
First Named Inventor: Brian K. Maples	Title: Nicking and Extension Amplification Reaction for the Exponential Amplification of Nucleic Acids	

APPLICANT HEREBY REQUESTS A FIRST-ACTION INTERVIEW IN THE ABOVE-IDENTIFIED APPLICATION. See Instruction Sheet on page 2.

1. The application must contain three (3) or fewer independent claims and twenty (20) or fewer total claims.

2. The application must not contain any multiple dependent claims.

3. By filing this request:

   Applicant is agreeing to make an election without traverse if the Office determines that the claims are not obviously directed to a single invention; and

   Applicant is agreeing not to request for a refund of the search fee and any excess claims fee paid in the application after the mailing or notification of the pre-interview communication prepared by the examiner.

4. Other attachments: ____________________

Signature /Ian J.S. Lodovice, Reg. No. 59,749/	Date October 15, 2014
Name (Print/Typed) Ian J. Lodovice	Registration Number 59,749

***Note**: Signatures of all the inventors or assignees of record of the entire interest or their representative(s) are required in accordance with 37 CFR 1.33 and 10.18. Please see 37 CFR 1.4(d) for the form of the signature. If necessary, submit multiple forms for more than one signature, see below*.*

☒ *Total of 1 forms are submitted.

The information is required to obtain or retain a benefit by the public which is to file (and by the USPTO to process) an application. Confidentiality is governed by 35 U.S.C. 122 and 37 CFR 1.11 and 1.14. This collection is estimated to take 12 hours to complete, including gathering, preparing, and submitting the completed application form to the USPTO. Time will vary depending upon the individual case. Any comments on the amount of time you require to complete this form and/or suggestions for reducing this burden, should be sent to the Chief Information Officer, U.S. Patent and Trademark Office, U.S. Department of Commerce, P.O. Box 1450, Alexandria, VA 22313-1450. DO NOT SEND FEES OR COMPLETED FORMS TO THIS ADDRESS. **SEND TO: Commissioner for Patents, P.O. Box 1450, Alexandria, VA 22313-1450.**

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PTO/SB/06 (09-11)
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PATENT APPLICATION FEE DETERMINATION RECORD Substitute for Form PTO-875	Application or Docket Number 14/067,620	Filing Date 10/30/2013	☐ To be Mailed

ENTITY: ☒ LARGE ☐ SMALL ☐ MICRO

APPLICATION AS FILED – PART I

FOR	NUMBER FILED (Column 1)	NUMBER EXTRA (Column 2)	RATE ($)	FEE ($)
☐ BASIC FEE (37 CFR 1.16(a), (b), or (c))	N/A	N/A	N/A	
☐ SEARCH FEE (37 CFR 1.16(k), (i), or (m))	N/A	N/A	N/A	
☐ EXAMINATION FEE (37 CFR 1.16(o), (p), or (q))	N/A	N/A	N/A	
TOTAL CLAIMS (37 CFR 1.16(i))	minus 20 =	*	X $ =	
INDEPENDENT CLAIMS (37 CFR 1.16(h))	minus 3 =	*	X $ =	
☐ APPLICATION SIZE FEE (37 CFR 1.16(s))	If the specification and drawings exceed 100 sheets of paper, the application size fee due is $310 ($155 for small entity) for each additional 50 sheets or fraction thereof. See 35 U.S.C. 41(a)(1)(G) and 37 CFR 1.16(s).			
☐ MULTIPLE DEPENDENT CLAIM PRESENT (37 CFR 1.16(j))				
* If the difference in column 1 is less than zero, enter "0" in column 2.			TOTAL	

APPLICATION AS AMENDED – PART II

AMENDMENT	10/15/2014	CLAIMS REMAINING AFTER AMENDMENT (Column 1)		HIGHEST NUMBER PREVIOUSLY PAID FOR (Column 2)	PRESENT EXTRA (Column 3)	RATE ($)	ADDITIONAL FEE ($)
	Total (37 CFR 1.16(i))	* 16	Minus	** 29	= 0	x $80 =	0
	Independent (37 CFR 1.16(h))	* 3	Minus	***3	= 0	x $420 =	0
	☐ Application Size Fee (37 CFR 1.16(s))						
	☐ FIRST PRESENTATION OF MULTIPLE DEPENDENT CLAIM (37 CFR 1.16(j))						
						TOTAL ADD'L FEE	**0**

AMENDMENT		CLAIMS REMAINING AFTER AMENDMENT (Column 1)		HIGHEST NUMBER PREVIOUSLY PAID FOR (Column 2)	PRESENT EXTRA (Column 3)	RATE ($)	ADDITIONAL FEE ($)
	Total (37 CFR 1.16(i))	*	Minus	**	=	X $ =	
	Independent (37 CFR 1.16(h))	*	Minus	***	=	X $ =	
	☐ Application Size Fee (37 CFR 1.16(s))						
	☐ FIRST PRESENTATION OF MULTIPLE DEPENDENT CLAIM (37 CFR 1.16(j))						
						TOTAL ADD'L FEE	

* If the entry in column 1 is less than the entry in column 2, write "0" in column 3.
** If the "Highest Number Previously Paid For" IN THIS SPACE is less than 20, enter "20".
*** If the "Highest Number Previously Paid For" IN THIS SPACE is less than 3, enter "3".
The "Highest Number Previously Paid For" (Total or Independent) is the highest number found in the appropriate box in column 1.

LIE
/LINDA A. WASHINGTON/

This collection of information is required by 37 CFR 1.16. The information is required to obtain or retain a benefit by the public which is to file (and by the USPTO to process) an application. Confidentiality is governed by 35 U.S.C. 122 and 37 CFR 1.14. This collection is estimated to take 12 minutes to complete, including gathering, preparing, and submitting the completed application form to the USPTO. Time will vary depending upon the individual case. Any comments on the amount of time you require to complete this form and/or suggestions for reducing this burden, should be sent to the Chief Information Officer, U.S. Patent and Trademark Office, U.S. Department of Commerce, P.O. Box 1450, Alexandria, VA 22313-1450. DO NOT SEND FEES OR COMPLETED FORMS TO THIS ADDRESS. **SEND TO: Commissioner for Patents, P.O. Box 1450, Alexandria, VA 22313-1450.**
If you need assistance in completing the form, call 1-800-PTO-9199 and select option 2.

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

Applicant	:	Ionian Technologies Inc.	Art Unit	:	1637
Serial No.	:	14/067,620	Examiner	:	Olayinka A. Oyeyemi
Filed	:	October 30, 2013	Conf. No.	:	4288
Title	:	NICKING AND EXTENSION AMPLIFICATION REACTION FOR THE EXPONENTIAL AMPLIFICATION OF NUCLEIC ACIDS			

MAIL STOP AMENDMENT
Commissioner for Patents
P.O. Box 1450
Alexandria, VA 22313-1450

INFORMATION DISCLOSURE STATEMENT

Please consider the references listed on the enclosed PTO-SB-08 or Disclosure Form. Foreign patent documents and non-patent literature are enclosed; cited U.S. patents and patent application publications will be provided on request. A copy of a communication from a foreign patent office in a counterpart application is also enclosed.

This statement is being filed within three months of the filing date of the application or before the receipt of a first Office Action on the merits. Please apply any necessary charges or credits to Deposit Account 06-1050, referencing the above attorney docket number.

Respectfully submitted,

Date: June 26, 2014

/Ian J.S. Lodovice, Reg. No. 59,749/
Ian J. Lodovice
Reg. No. 59,749

Customer Number 26161
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Telephone: (617) 956-5972
Facsimile: (877) 769-7945

23245596.doc

Substitute Disclosure Form U.S. Department of Commerce Patent and Trademark Office	Attorney Docket No. 30171-0025002	Application No. 14/067,620
Information Disclosure Statement by Applicant (Use several sheets if necessary)	First Named Inventor Brian K. Maples	
(37 CFR §1.98(b))	Filing Date October 30, 2013	Group Art Unit 1637

U.S. Patent Documents

Examiner Initial	Desig. ID	Document Number	Publication Date	Patentee	Class	Subclass	Filing Date If Appropriate
	1						

Foreign Patent Documents or Published Foreign Patent Applications

Examiner Initial	Desig. ID	Document Number	Publication Date	Country or Patent Office	Class	Subclass	Translation	
							Yes	No
	2							

Other Documents (include Author, Title, Date, and Place of Publication)

Examiner Initial	Desig. ID	Document
	3	Copy of English translation of Chinese Third Office action, for corresponding Chinese application CN 200880105424.7. dated February 8, 2014
	4	Notification of the Final Rejection for corresponding Chinese application CN 200880105424.7. dated June 5, 2014
	5	
	6	

Examiner Signature	Date Considered
EXAMINER: Initials citation considered. Draw line through citation if not in conformance and not considered. Include copy of this form with next communication to applicant.	

Substitute Disclosure Form

Electronic Acknowledgement Receipt

EFS ID:	19428900
Application Number:	14067620
International Application Number:	
Confirmation Number:	4288
Title of Invention:	Nicking and Extension Amplification Reaction for the Exponential Amplification of Nucleic Acids
First Named Inventor/Applicant Name:	Brian K. Maples
Customer Number:	26161
Filer:	Ian J.S. Lodovice/Mary Florczak
Filer Authorized By:	Ian J.S. Lodovice
Attorney Docket Number:	30171-0025002 / ITI-001
Receipt Date:	26-JUN-2014
Filing Date:	30-OCT-2013
Time Stamp:	19:50:40
Application Type:	Utility under 35 USC 111(a)

Payment information:

Submitted with Payment	no

File Listing:

Document Number	Document Description	File Name	File Size(Bytes)/ Message Digest	Multi Part /.zip	Pages (if appl.)
1	Information Disclosure Statement (IDS) Form (SB08)	301710025002IDS.pdf	114043 6be920bb848080be6fbecd8518e6696bc5c929a2	no	2

Warnings:

Information:

This is not an USPTO supplied IDS fillable form					
2	Non Patent Literature	30171CNFinalRej.pdf	71953 f26d85fe7a39514576187a32aa6610604592 3466	no	10
Warnings:					
Information:					
3	Non Patent Literature	30171ThirdOA.pdf	185124 b316ae28e1da634fa4730d53623fe3363fa0 f844	no	10
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Information:					
		Total Files Size (in bytes):	371120		

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New Applications Under 35 U.S.C. 111
If a new application is being filed and the application includes the necessary components for a filing date (see 37 CFR 1.53(b)-(d) and MPEP 506), a Filing Receipt (37 CFR 1.54) will be issued in due course and the date shown on this Acknowledgement Receipt will establish the filing date of the application.

National Stage of an International Application under 35 U.S.C. 371
If a timely submission to enter the national stage of an international application is compliant with the conditions of 35 U.S.C. 371 and other applicable requirements a Form PCT/DO/EO/903 indicating acceptance of the application as a national stage submission under 35 U.S.C. 371 will be issued in addition to the Filing Receipt, in due course.

New International Application Filed with the USPTO as a Receiving Office
If a new international application is being filed and the international application includes the necessary components for an international filing date (see PCT Article 11 and MPEP 1810), a Notification of the International Application Number and of the International Filing Date (Form PCT/RO/105) will be issued in due course, subject to prescriptions concerning national security, and the date shown on this Acknowledgement Receipt will establish the international filing date of the application.

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

Applicant	:	Ionian Technologies Inc.	Art Unit	:	1637
Serial No.	:	14/067,620	Examiner	:	Olayinka A. Oyeyemi
Filed	:	October 30, 2013	Conf. No.	:	4288
Title	:	NICKING AND EXTENSION AMPLIFICATION REACTION FOR THE EXPONENTIAL AMPLIFICATION OF NUCLEIC ACIDS			

MAIL STOP AMENDMENT
Commissioner for Patents
P.O. Box 1450
Alexandria, VA 22313-1450

INFORMATION DISCLOSURE STATEMENT

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This statement is being filed within three months of the filing date of the application or before the receipt of a first Office Action on the merits. Please apply any necessary charges or credits to Deposit Account 06-1050, referencing the above attorney docket number.

Respectfully submitted,

Date: May 23, 2014

/Ian J.S. Lodovice, Reg. No. 59,749/
Ian J. Lodovice
Reg. No. 59,749

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Substitute Disclosure Form U.S. Department of Commerce Patent and Trademark Office	Attorney Docket No. 30171-0025002	Application No. 14/067,620
Information Disclosure Statement by Applicant (Use several sheets if necessary)	First Named Inventor Brian K. Maples	
(37 CFR §1.98(b))	Filing Date October 30, 2013	Group Art Unit 1637

U.S. Patent Documents

Examiner Initial	Desig. ID	Document Number	Publication Date	Patentee	Class	Subclass	Filing Date If Appropriate
	1						

Foreign Patent Documents or Published Foreign Patent Applications

Examiner Initial	Desig. ID	Document Number	Publication Date	Country or Patent Office	Class	Subclass	Translation Yes	Translation No
	2	EP2660333	11/06/2013	Europe				
	3	EP2657350	10/30/2013	Europe				
	4	WO2007/028833	03/15/2007	WIPO				
	5	WO94/03635	02/17/1997	WIPO				
	6							

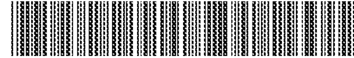
Other Documents (include Author, Title, Date, and Place of Publication)

Examiner Initial	Desig. ID	Document
	7	Sequence of vector pUC19, downloaded from http://genome-www.stanford.edu/vectordb/vector_descrip/COMPLET... On March 27, 2014
	8	Notice of Opposition in corresponding EP Application No. 08781827.4, dated May 6, 2014, pages 1-36
	9	

Examiner Signature	Date Considered
EXAMINER: Initials citation considered. Draw line through citation if not in conformance and not considered. Include copy of this form with next communication to applicant.	

Substitute Disclosure Form

(19)



(11) **EP 2 657 350 A1**

(12) **EUROPEAN PATENT APPLICATION**

(43) Date of publication:
30.10.2013 Bulletin 2013/44

(51) Int Cl.:
C12Q 1/68 (2006.01)

(21) Application number: **12195331.9**

(22) Date of filing: **14.07.2008**

(84) Designated Contracting States:
AT BE BG CH CY CZ DE DK EE ES FI FR GB GR HR HU IE IS IT LI LT LU LV MC MT NL NO PL PT RO SE SI SK TR
Designated Extension States:
AL

(30) Priority: **14.07.2007 US 778018**

(62) Document number(s) of the earlier application(s) in accordance with Art. 76 EPC:
08781827.4 / 2 181 196

(71) Applicant: **Ionian Technologies, Inc.**
San Diego, CA 92121 (US)

(72) Inventors:
- **Maples, Brian K.**
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Remarks:
•This application was filed on 03-12-2012 as a divisional application to the application mentioned under INID code 62.
•Claims filed after date of filing / after date of receipt of divisional application (Rule 68(4) EPC).

(54) **Nicking and extension amplification reaction for the exponential amplification of nucleic acids**

(57) The invention is in general directed to the rapid exponential amplification of short DNA or RNA sequences at a constant temperature.

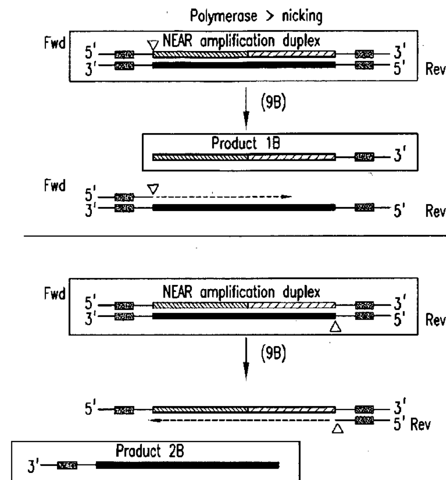

FIG.1C

Printed by Jouve, 75001 PARIS (FR)

EP 2 657 350 A1

Description

Field of the Invention

5 **[0001]** The invention is in general directed to the rapid exponential amplification of short DNA or RNA sequences at a constant temperature.

Related Applications

10 **[0002]** Priority is claimed to U.S. Patent Application serial number 11/778,018, filed July 14, 2007, and entitled Nicking and Extension Amplification Reaction for the Exponential Amplification of Nucleic Acids, which is referred to and incorporated herein by reference in its entirety.

Background

15

[0003] The field of *in vitro* diagnostics is quickly expanding as the need for systems that can rapidly detect the presence of harmful species or determine the genetic sequence of a region of interest is increasing exponentially. Current molecular diagnostics focus on the detection of biomarkers and include small molecule detection, immuno-based assays, and nucleic acid tests. The built-in specificity between two complementary or substantially complementary nucleic acid
20 strands allows for fast and specific recognition using unique DNA or RNA sequences, the simplicity of which makes a nucleic acid test an attractive prospect. Identification of bacterial and viral threat agents, genetically modified food products, and single nucleotide polymorphisms for disease management are only a few areas where the advancement of these molecular diagnostic tools becomes extremely advantageous. To meet these growing needs, nucleic acid amplification technologies have been developed and tailored to these needs of specificity and sensitivity.

25 **[0004]** Historically, the most common amplification technique is the polymerase chain reaction (PCR), which has in many cases become the gold standard for detection methods because of its reliability and specificity. This technique requires the cycling of temperatures to proceed through the steps of denaturation of the dsDNA, annealing of short oligonucleotide primers, and extension of the primer along the template by a thermostable polymerase. Though many new advances in engineering have successfully shortened these reaction times to 20- 30 minutes, there is still a steep
30 power requirement to meet the needs of these thermocycling units.

[0005] Various isothermal amplification techniques have been developed to circumvent the need for temperature cycling. From this demand, both DNA and RNA isothermal amplification technologies have emerged.

[0006] Transcription- Mediated Amplification (TMA) employs a reverse transcriptase with RNase activity, an RNA polymerase, and primers with a promoter sequence at the 5'end. The reverse transcriptase synthesizes cDNA from the
35 primer, degrades the RNA target, and synthesizes the second strand after the reverse primer binds. RNA polymerase then binds to the promoter region of the dsDNA and transcribes new RNA transcripts which can serve as templates for further reverse transcription. The reaction can produce a billion fold amplification in 20- 30 minutes. This system is not as robust as other DNA amplification techniques and is therefore, not a field- deployable test due to the ubiquitous presence of RNAases outside of a sterile laboratory. This amplification technique is very similar to Self- Sustained
40 Sequence Replication (3 SR) and Nucleic Acid Sequence Based Amplification (NASBA), but varies in the enzymes employed.

[0007] Single Primer Isothermal Amplification (SPIA) also involves multiple polymerases and RNaseH. First, a reverse transcriptase extends a chimeric primer along an RNA target. RNaseH degrades the RNA target and allows a DNA polymerase to synthesize the second strand of cDNA. RNaseH then degrades a portion of the chimeric primer to release
45 a portion of the cDNA and open a binding site for the next chimeric primer to bind and the amplification process proceeds through the cycle again. The linear amplification system can amplify very low levels of RNA target in roughly 3.5 hrs.

[0008] The Q-Beta replicase system is a probe amplification method. A probe region complementary or substantially complementary to the target of choice is inserted into MDV-1 RNA, a naturally occurring template for Q-Beta replicase. Q-Beta replicates the MDV-1 plasmid so that the synthesized product is itself a template for Q-Beta replicase, resulting
50 in exponential amplification as long as the there is excess replicase to template. Because the Q-Beta replication process is so sensitive and can amplify whether the target is present or not, multiple wash steps are required to purge the sample of non-specifically bound replication plasmids. The exponential amplification takes approximately 30 minutes; however, the total time including all wash steps is approximately 4 hours.

[0009] Numerous isothermal DNA amplification technologies have been developed as well. Rolling circle amplification
55 (RCA) was developed based on the natural replication of plasmids and viruses. A primer extends along a circular template resulting in the synthesis of a single-stranded tandem repeat. Capture, washing, and ligation steps are necessary to preferentially circularize the template in the presence of target and reduce background amplification. Ramification amplification (RAM) adds cascading primers for additional geometric amplification. This technique involves amplification of

non-specifically sized strands that are either double or single-stranded.

[0010] Helicase- dependent amplification (HDA) takes advantage of a thermostable helicase (Tte- UvrD) to unwind dsDNA to create single- strands that are then available for hybridization and extension of primers by polymerase. The thermostable HDA method does not require the accessory proteins that the non- thermostable HDA requires. The reaction can be performed at a single temperature, though an initial heat denaturation to bind the primers generates more product. Reaction times are reported to be over 1 hour to amplify products 70- 120 base pairs in length.

[0011] Loop mediated amplification (LAMP) is a sensitive and specific isothermal amplification method that employs a thermostable polymerase with strand displacement capabilities and four or more primers. The primers are designed to anneal consecutively along the target in the forward and reverse direction. Extension of the outer primers displaces the extended inner primers to release single strands. Each primer is designed to have hairpin ends that, once displaced, snap into a hairpin to facilitate self-priming and further polymerase extension. Additional loop primers can decrease the amplification time, but complicates the reaction mixture. Overall, LAMP is a difficult amplification method to multiplex, that is, to amplify more than one target sequence at a time, although it is reported to be extremely specific due to the multiple primers that must anneal to the target to further the amplification process. Though the reaction proceeds under isothermal conditions, an initial heat denaturation step is required for double-stranded targets. Amplification proceeds in 25 to 50 minutes and yields a ladder pattern of various length products.

[0012] Strand displacement amplification (SDA) was developed by Walker et.al. in 1992. This amplification method uses two sets of primers, a strand displacing polymerase, and a restriction endonuclease. The bumper primers serve to displace the initially extended primers to create a single-strand for the next primer to bind. A restriction site is present in the 5' region of the primer. Thiol-modified nucleotides are incorporated into the synthesized products to inhibit cleavage of the synthesized strand. This modification creates a nick site on the primer side of the strand, which the polymerase can extend. This approach requires an initial heat denaturation step for double-stranded targets. The reaction is then run at a temperature below the melting temperature of the double-stranded target region. Products 60 to 100 bases in length are usually amplified in 30-45 minutes using this method.

[0013] These and other amplification methods are discussed in, for example, VanNess, J, et al., PNAS 2003, vol 100, no 8, p 4504-4509; Tan, E., et al., Anal. Chem. 2005, 77, 7984-7992; Lizard, P., et al., Nature Biotech. 1998, 6, 1197-1202; Notomi, T., et al., NAR 2000, 28, 12, e63; and Kurn, N., et al., Clin. Chem. 2005, 51:10, 1973-1981. Other references for these general amplification techniques include, for example, U.S. Patent Serial Nos. 7112423; 5455166; 5712124; 5744311; 5916779; 5556751; 5733733; 5834202; 5354668; 5591609; 5614389; 5942391; and U.S. patent publication numbers US20030082590; US20030138800; US20040058378; and US20060154286.

Summary

[0014] Provided herein are methods of amplifying nucleic acid target sequences that rely on nicking and extension reactions to amplify shorter sequences in a quicker timeframe than traditional amplification reactions, such as, for example, strand displacement amplification reactions. Embodiments of the invention include, for example, reactions that use only two templates to amplify a target sequence, one or two nicking enzymes, and a polymerase, under isothermal conditions. In exemplary embodiments, the polymerase and the nicking enzyme are thermophilic, and the reaction temperature is significantly below the melting temperature of the hybridized target region. The nicking enzyme nicks only one strand in a double-stranded duplex, so that incorporation of modified nucleotides is not necessary as in the case of conventional strand displacement amplification. An initial heat denaturation step is not required for the methods of the present invention. Due to the simplicity of the reaction, in exemplary embodiments, the reaction is very easy to perform, requires no special equipment, such as a thermocycler, and can amplify 20-30mer products 10^8 to 10^{10} fold from genomic DNA in only about 2.5 to about 10 minutes. Furthermore, in other exemplary embodiments, the method is able to amplify RNA without a separate reverse transcription step.

[0015] Thus, provided in a first embodiment of the present invention is a method for amplifying a double- stranded nucleic acid target sequence, comprising contacting a target DNA molecule comprising a double- stranded target sequence having a sense strand and an antisense strand, with a forward template and a reverse template, wherein said forward template comprises a nucleic acid sequence comprising a recognition region at the 3' end that is complementary or substantially complementary to the 3' end of the target sequence antisense strand; a nicking enzyme binding site and a nicking site upstream of said recognition region, and a stabilizing region upstream of said nicking enzyme binding site and said nicking site; said reverse template comprises a nucleotide sequence comprising a recognition region at the 3' end that is complementary or substantially complementary to the 3' end of the target sequence sense strand, a nicking enzyme binding site and a nicking site upstream of said recognition region, and a stabilizing region upstream of said nicking enzyme binding site and said nicking site; providing a first nicking enzyme that is capable of nicking at the nicking site of said forward template, and does not nick within said target sequence; providing a second nicking enzyme that is capable of nicking at the nicking site of said reverse template and does not nick within said target sequence; and providing a DNA polymerase; under conditions wherein amplification is performed by multiple cycles of said polymerase extending

said forward and reverse templates along said target sequence producing a double- stranded nicking site, and said nicking enzymes nicking at said nicking sites, producing an amplification product.

[0016] In certain embodiments of the invention, the DNA polymerase is a thermophilic polymerase. In other examples of the invention, the polymerase and said nicking enzymes are stable at temperatures up to 37°C, 42°C, 60°C, 65°C, 70°C, 75°C, 80°C, or 85°C. In certain embodiments, the polymerase is stable up to 60°C. The polymerase may, for example, be selected from the group consisting of Bst (large fragment), 9°N, $Vent_R$® (exo-) DN Polymerase, Therminator, and Therminator II.

[0017] The nicking enzyme may, for example, nick upstream of the nicking enzyme binding site, or, in exemplary embodiments, the nicking enzyme may nick downstream of the nicking enzyme binding site. In certain embodiments, the forward and reverse templates comprise nicking sites recognized by the same nicking enzyme and said first and said second nicking enzyme are the same. The nicking enzyme may, for example, be selected from the group consisting of Nt.BspQI, Nb.BbvCi, Nb.BsmI, Nb.BsrDI, Nb.BtsI, Nt.AlwI, Nt.BbvCI, Nt.BstNBI, Nt.CviPII, Nb.BpuIOI, and Nt.Bpu10I.

[0018] In certain aspects of the present invention, the target sequence comprises from 1 to 5 nucleotides more than the sum of the nucleotides of said forward template recognition region and said reverse template recognition region.

[0019] The DNA molecule may be, for example, genomic DNA. The DNA molecule may be, for example, selected from the group consisting of plasmid, mitochondrial, and viral DNA. In certain embodiments, the forward template is provided at the same concentration as the reverse template. In other examples, the forward template is provided at a ratio to the reverse template at the range of ratios of 1:100 to 100:1.

[0020] In other examples of the invention, the method further comprises the use of a second polymerase. The amplification may be, for example, conducted at a constant temperature. This temperature may be, for example, between 54°C and 60°C. As to the length of time for the reaction to take place, in certain examples, the amplification reaction is held at constant temperature for 1 to 10 minutes.

[0021] The present invention further comprises detecting the amplification product, for example, by a method selected from the group consisting of gel electrophoresis, mass spectrometry, SYBR I fluorescence, SYBR II fluorescence, SYBR Gold, Pico Green, TOTO-3, intercalating dye detection, FRET, molecular beacon detection, surface capture, capillary electrophoresis, incorporation of labeled nucleotides to allow detection by capture, fluorescence polarization, and lateral flow capture. The amplification products may be, for example, detected using a solid surface method, for example, where at least one capture probe is immobilized on the solid surface that binds to the amplified sequence.

[0022] The present invention may be used for multiplex amplification. Thus, for example, in certain embodiments of the present invention at least two target sequences are capable of being amplified. By "capable of being amplified" is meant the amplification reaction comprises the appropriate templates and enzymes to amplify at least two target sequences. Thus, for example, the amplification reaction may be prepared to detect at least two target sequences, but only one of the target sequences may actually be present in the sample being tested, such that both sequences are capable of being amplified, even though only one sequence may actually be amplified. Or, where two target sequences are present, the amplification reaction may result in the amplification of both of the target sequences. The multiplex amplification reaction may result in the amplification of one, some, or all, of the target sequences for which it comprises the appropriate templates and enzymes.

[0023] At least one of the templates, for example, may comprise a spacer, a blocking group, or a modified nucleotide.

[0024] Also provided as an embodiment of the present invention is a method for amplifying a single-stranded nucleic acid target sequence, comprising contacting a target nucleic acid comprising a single-stranded target sequence with a reverse template, wherein said reverse template comprises a nucleotide sequence comprising a recognition region at the 3' end that is complementary or substantially complementary to the 3' end of the target sequence, a nicking enzyme binding site and a nicking site upstream of said recognition region, and a stabilizing region upstream of said nicking enzyme binding site and said nicking site; providing a first nicking enzyme that is capable of nicking at the nicking site of said reverse template, and does not nick within said target sequence; providing a DNA polymerase under conditions wherein said polymerase extends said reverse template along said target sequence; contacting said extended reverse template with a forward template, wherein said forward template comprises a recognition region at the 3' end that is identical to the 5' end of the target sequence a nicking enzyme binding site and a nicking site upstream of said recognition region, and a stabilizing region upstream of said nicking enzyme binding site and said nicking site; providing a second nicking enzyme that is capable of nicking at the nicking site of said forward template and does not nick within said target sequence; under conditions wherein amplification is performed by multiple cycles of said polymerase extending said forward and reverse templates along said target sequence producing a double-stranded nicking site, and said nicking enzymes nicking at said nicking sites, producing an amplification product.

[0025] Those of ordinary skill in the art understand that the examples presented herein relating to the amplification of a double-stranded nucleic acid target sequence and the detection of the amplified product also apply to the amplification of a single-stranded nucleic acid target sequence and the detection of the amplified product. Furthermore, in examples of the present invention, the target sequence may be, for example, RNA, for example, but not limited to, messenger RNA (mRNA), ribosomal RNA (rRNA), viral RNA, microRNA, a microRNA precursor, or siRNA. In exemplary embodiments

of the present invention, for example, where the target sequence is RNA, the polymerase has reverse transcription activity. In yet other examples of the present invention, the target sequence is DNA, such as, for example, genomic DNA, or for example, the target sequence is selected from the group consisting of plasmid, mitochondrial, and viral DNA, or even a PCR product.

[0026] Where the method, in accordance with the present invention, involves the use of more than one polymerase, in exemplary embodiments at least one of the polymerases may have reverse transcriptase activity.

[0027] In other embodiments of the present invention, a set of oligonucleotide templates is provided, comprising a first template for nucleic acid amplification, comprising a recognition region at the 3' end that is complementary or substantially complementary to the 3' end of a target sequence antisense strand; a nicking enzyme binding site and a nicking site upstream of said recognition region; and a stabilizing region upstream of said nicking enzyme binding site and said nicking site; and a second template for nucleic acid amplification, comprising a recognition region at the 3' end that is identical to the 5' of said target sequence antisense strand; nicking enzyme binding site and a nicking site upstream of said recognition region; and a stabilizing region upstream of said nicking enzyme binding site and said nicking site; wherein said target sequence comprises from 1 to 5 spacer bases between said 3' end of the antisense strand and said 5' end of said antisense strand that do not bind to either template.

[0028] In yet other embodiments, a kit is provided for following the methods of the present invention for nucleic acid amplification, comprising a DNA polymerase; a first template for nucleic acid amplification, comprising a recognition region at the 3' end that is complementary or substantially complementary to the 3' end of a target sequence antisense strand; a nicking enzyme binding site and a nicking site upstream of said recognition region; and a stabilizing region upstream of said nicking enzyme binding site and said nicking site; a second template for nucleic acid amplification, comprising a recognition region at the 3' end that is complementary or substantially complementary to the 3' end of a target sequence sense strand; nicking enzyme binding site and a nicking site upstream of said recognition region; and a stabilizing region upstream of said nicking enzyme binding site and said nicking site; one or two thermostable nicking enzymes, wherein either one enzyme is capable of nicking at the nicking site of said first and said second templates, or a first enzyme is capable of nicking at the nicking site of said first primer and a second enzyme is capable of nicking at the enzyme site of said second primer.

[0029] The kit may, for example, provide said polymerase, nicking enzymes, and templates in a container. The kit may provide, for example, said polymerase, nicking enzymes, and templates in two containers. In certain examples, the polymerase and nicking enzymes are in a first container, and said templates are in a second container. In certain examples, the polymerase and nicking enzymes are lyophilized. The kit may, for example, further comprise instructions for following the amplification methods of the present invention. The kit may, for example, further comprise a cuvette. The kit may, for example, further comprise a lateral flow device or dipstick. The lateral flow device or dipstick may, for example, further comprise a capture probe, wherein said capture probe binds to amplified product. The kit may, for example, further comprise a detector component, for example, one selected from the group consisting of a fluorescent dye, colloidal gold particles, latex particles, a molecular beacon, polystyrene beads, and the like. In other examples, at least one of the templates of the kit may comprise a spacer, blocking group, or a modified nucleotide.

[0030] Deoxynucleoside triphosphates (dNTPs) are included in the amplification reaction. One or more of the dNTPs may be modified, or labeled, as discussed herein, however, the use of modified NTPs is not required in the present method. Nucleotides are designated as follows. A ribonucleoside triphosphate is referred to as NTP or rNTP; wherein N can be A, G, C, U or m5U to denote specific ribonucleotides. Deoxynucleoside triphosphate substrates are indicated as dNTPs, wherein N can be A, G, C, T, or U. Throughout the text, monomeric nucleotide subunits may be denoted as A, G, C, or T with no particular reference to DNA or RNA.

[0031] In another embodiment, a method is provided for nucleic acid amplification comprising forming a mixture of a target nucleic acid comprising a double-stranded target sequence having a sense strand and an antisense strand; a forward template comprising a nucleic acid sequence comprising a recognition region at the 3' end that is complementary or substantially complementary to the 3' end of the target sequence antisense strand; a nicking enzyme binding site and a nicking site upstream of said recognition region, and a stabilizing region upstream of said nicking enzyme binding site and said nicking site; a reverse template comprising a nucleotide sequence comprising a recognition region at the 3' end that is complementary or substantially complementary to the 3' end of the target sequence sense strand, a nicking enzyme binding site and a nicking site upstream of said recognition region and a stabilizing region upstream of said nicking enzyme binding site and said nicking site; a first nicking enzyme that is capable of nicking at the nicking site of said forward template, and does not nick within said target sequence; a second nicking enzyme that is capable of nicking at the nicking site of said reverse template and does not nick within said target sequence; and a thermophilic polymerase under conditions wherein amplification is performed by multiple cycles of said polymerase extending said forward and reverse templates along said target sequence producing a double-stranded nicking site, and said nicking enzymes nicking at said nicking sites, producing an amplification product. In certain embodiments, the nicking enzyme binding sites on the forward and reverse templates are recognized by the same nicking enzyme, and only one nicking enzyme is used for the reaction.

[0032] In another embodiment, a method is provided for nucleic acid amplification comprising forming a mixture of a target nucleic acid comprising a single-stranded target sequence; a reverse template, wherein said reverse template comprises a nucleotide sequence comprising a recognition region at the 3' end that is complementary or substantially complementary to the 3' end of the target sequence, a nicking enzyme binding site and a nicking site upstream of said recognition region, and a stabilizing region upstream of said nicking enzyme binding site and said nicking site; a first nicking enzyme that is capable of nicking at the nicking site of said reverse template, and does not nick within said target sequence; a thermophilic polymerase under conditions wherein said polymerase extends said reverse template along said target sequence; a forward template, wherein said forward template comprises a nucleic acid sequence comprising a recognition region at the 3' end that is identical or substantially identical to the 5'end of the target sequence; and a second nicking enzyme that is capable of nicking at the nicking site of said forward template and does not nick within said target sequence; under conditions wherein amplification is performed by multiple cycles of said polymerase extending said forward and reverse templates along said target sequence producing a double-stranded nicking site, and said nicking enzymes nicking at said nicking sites, producing an amplification product. In certain embodiments, the nicking enzyme binding sites on the forward and reverse templates are recognized by the same nicking enzyme, and only one nicking enzyme is used for the reaction.

[0033] In other embodiments of the invention are provided methods for the separation of amplified nucleic acids obtained by the amplification methods of the invention. In yet further embodiments of the invention are provided methods for detecting and/or analyzing the amplified nucleic acids obtained by the amplification methods of the invention, including, for example, methods using SYBR I, II, SYBR Gold, Pico Green, TOTO-3, and most intercalating dyes, molecular beacons, FRET, surface capture using immobilized probes with fluorescence, electrochemical, or colorimetric detection, mass spectrometry, capillary electrophoresis, the incorporation of labeled nucleotides to allow detection by capture or fluorescence polarization, lateral flow, and other methods involving capture probes.

[0034] Methods using capture probes for detection include, for example, the use of a nucleic acid molecule (the capture probe) comprising a sequence that is complementary to, or substantially complementary to, an amplification product strand such that the capture probe binds to amplified nucleic acid. The probe may be linked to a detectable label in certain embodiments, and amplification product may be detected based on the detectable label of the probe specifically hybridized to the amplification product. The reaction may, for example, further comprise an antibody directed against a molecule incorporated into or attached to the capture probe. Or, for example, the capture probe, or a molecule that binds to the capture probe, may incorporate, for example, an enzyme label, for example, peroxidase, alkaline phosphatase, or beta-galactosidase, a fluorescent label, such as, for example, fluorescein or rhodamine, or, for example, other molecules having chemiluminescent or bioluminescent activity. In some embodiments, the probe is linked to a solid support, and amplification product strands may be specifically immobilized to the capture probe linked to the solid support under conditions known and selected by the person of ordinary skill in the art. In the latter embodiments, solid support-immobilized amplification product may be subjected to processing steps, such as washing, ion exchange, release from the solid support, or other processing steps. An amplification product may be detected when immobilized to a solid support in some embodiments. The embodiments of the present invention also comprise combinations of these detection and analysis methods.

Brief Description of the Drawings

[0035] **Figures 1A-D** are graphic drawings depicting mechanisms of the reactions of the present invention. Figure 1D is a legend for Figure 1.

[0036] **Figure 2.** 20% polyacrylamide gel of reaction products from a DNA NEAR™ assay.

[0037] A reaction following the present methods was run for 2.5 minutes at 56 °C, then heat denatured at 94 °C for 4 minutes. Six microliters of the reaction was run on a 20% polyacrylamide gel at 160V for ~2.5 hrs. The gel was stained with SYBR II gel stain. Lane 1: no target control for 25mer assay. Lane 2: no target control for 27mer assay. Lane 3: for 25mer assay with 3.5E+5 copies of genomic *Bacillus subtilis* DNA. Lane 4: for 27mer assay with 1.1E+6 copies of genomic *Bacillus subtilis* DNA.

[0038] **Figure 3.** 20% polyacrylamide gel of reaction products from an RNA assay using the present methods.

[0039] The reaction was run for 12 minutes at 56 °C, then heat denatured at 94 °C for 4 minutes. Six microliters of the reaction was run on a 20% polyacrylamide gel at 160V for about 2.5 hrs. The gel was stained with SYBR II gel stain. Lane 1 and 2: reaction for 25mer assay with 1E+6 copies of Ebola Armored RNA (Ambion). Lane 3 and 4: reaction no target control for 25mer assay. 25mer reaction products are outlined in the white box.

[0040] **Figure 4.** Mass Spectrum of *Bacillus anthracis* DNA assay products.

[0041] A) 0 copies of target or B) 5E+5 copies of genomic DNA added to the reaction. The reaction was run for 10 minutes, then heat denatured at 94 °C for 4 minutes. Ten microliters of sample was injected into the LC/ESI- MS. The (-4) charge state of the 26mer product and its complementary sequence are outlined in a black box. The smaller adjacent peaks are the sodium adducts of the main product.

[0042] **Figure 5.** Mass Spectrum of MS2 genomic RNA assay products. A) 0 copies of target, B) 1E+6 copies of MS2 genomic RNA, or C) 1E+6 copies of synthetic target DNA added to the reaction. The reaction was run for 10 minutes, then heat denatured at 94 °C for 4 minutes. Ten microliters of sample was injected into the LC/ESI-MS. The (-4) charge state of the 27mer product and its complement sequence are outlined in a black box. The smaller adjacent peaks are the sodium adducts of the main product.

[0043] **Figure 6.** Real-time detection of amplification using intercalating fluorescent dyes.

[0044] Real- time amplification of *Yersinia pestis* genomic DNA at 500 copies (squares) compared to the no target control (NTC, open triangles) . The reaction was run for 10 minutes at 58 °C and monitored by the real- time fluorescence with SYBR II (n = 5) .

[0045] **Figure 7.** Real-time detection of amplification using fluorescence resonance energy transfer (FRET).

[0046] Real- time amplification of *Yersinia pestis* synthetic DNA at 10, 000 copies (squares) compared to the no target control (NTC, open triangles) . The reaction was run for 10 minutes at 57 °C, n = 3.

[0047] **Figure 8.** Francisella *tularensis* DNA amplification detected in real-time using molecular beacons.

[0048] Either 0 copies (open triangles) or 1E+5 copies (squares) were added to the reaction mix and run for 10 minutes at 57.5 °C.

[0049] **Figure 9.** False positive rate testing results comparing average AUC values.

[0050] Error bars denote one standard deviation. *Bacillus subtilis* assays were run for 10 min at 55 °C in the presence and absence of *Bacillus subtilis* genomic DNA. Enzymes were heat denatured at 94 °C for 4 min. A 10 μL sample was injected into the LC/ESI-MS and the area under the curve (AUC) of the product peaks were analyzed. True Positives contained 10,000 copies of *Bacillus subtilis* DNA along with 990,000 copies of near neighbor (*Bacillus thuringiensis*) DNA. True Negatives contained 10,000 copies of *E. coli* DNA with 990,000 copies of near neighbor DNA, and water negatives contained no DNA as a control.

[0051] **Figure 10.** Replication study using molecular beacon detection with different operators performing the experiments on two different days.

[0052] The reaction was run for 10 minutes at 57.5 °C (in the presence and absence of 500 copies of *Francisella tularensis* genomic DNA) with a 4 min heat kill at 94 °C. 300nM molecular beacon was added and monitored at 45, 50, and 57 °C (n = 24).

[0053] **Figure 11.** Sensitivity of the reaction using molecular beacon detection.

[0054] The assay was run for 10 minutes 57.5 °C. The reaction was stopped with a 4 min heat inactivation step at 94 °C. 300nM molecular beacon was added and the fluorescence was monitored at 57.5 °C (n = 3). Fluorescence was monitored for beacon opening in the presence of reactions amplified with 1E+6, 5E+5, 5E+4, 5E+2, 50, and 0 (NTC) input copies of *Francisella tularensis* genomic DNA, and compared to the background fluorescence of the beacon alone (MB).

[0055] **Figure 12.** Final concentration of amplified products in the NEAR reaction.

[0056] The NEAR ™ reaction was run for 10 min at 55 °C with varying copies of *Bacillus subtilis* genomic DNA. The reaction was stopped with a heat inactivation step at 94 °C for 4 minutes. A 10 microliter sample was injected into the LC/ESI-MS and the AUC of the product peak at 1944 Daltons was analyzed and compared to a standard curve.

[0057] **Figure 13.** Correlation of the input RNA target copy number to the final concentration of amplified products.

[0058] The Ebola NEAR ™ assay was run for 12 min at 55 °C with varying copies of synthetic RNA corresponding to the Ebola genome DNA. The reaction was stopped with a heat inactivation step at 94 °C for 4 minutes. A 10 microliter sample was injected into the **LC/ESI-MS** and the AUC of the product peak at 1936 Daltons was analyzed and compared to the standard curve of AUC values. (n = 3)

[0059] **Figure 14.** Mass spec product analysis demonstrating NEAR reaction specificity.

[0060] The *Bacillus anthracis* NEAR™ reaction was run in the presence of a dilution of copies of Bacillus thuringiensis for 10 min at 56 °C (n = 3), then heat denatured at 94°C for 4 minutes. A 10 μL sample was injected into the LC/ESI-MS and AUC values of product peaks analyzed.

[0061] **Figure 15.** The effect of an interferent panel on amplification.

[0062] *Bacillus subtilis* DNA reactions were run for 10 min at 55 °C and heated to 94 °C for 4 minutes to stop the reaction. Reactions were run in triplicate in the presence 1E+5 copies of *Bacillus subtilis* genomic DNA ("_1E +5") or with no target DNA present ("_0"). Sample x is the control assay with no interferent added. Interferents A through F were added at 50% reaction volume to the *Bacillus subtilis* assay. The AUC of mass spec product peaks were analyzed using a two-way ANOVA and Bonferroni t-test. (Key: A = none; B = House dust, skim milk; C = AZ test dust, humic acid; D = Diesel soot; E = Skim milk; F = Mold spores)

[0063] **Figure 16.** Gel electrophoresis results for the *Bacillus subtilis / Bacillus anthracis* DNA duplex reaction.

[0064] The NEAR™ reaction including templates specific for both *Bacillus subtilis (Bs)* and *Bacillus anthracis (Ba)* DNA was run in the absence of target DNA (negative), in the presence of *Bacillus subtilis* only (positive for 27mer product), and in the presence of both *Bacillus subtilis* and *Bacillus anthracis* (positive for 27mer and 25mer product respectively) . The target copy number used in this assay was 500, 000 copies. The assay was run for 10 min at 57 °C.

Templates varied in concentration between the assays to control the amplification (100nM for *Bacillus anthracis* and 50 nM for *Bacillus subtilis)* . Samples were run on a 20% polyacrylamide gel at 160 V for about 2 hours. The gel was stained with SYBR II fluorescent dye and imaged. The fluorescent bands were quantified and analyzed as the integrated optical density (IOD) (n = 8) .

[0065] **Figure 17.** Specificity results for the *Bacillus subtilis* / *Bacillus anthracis* DNA duplex reaction shown by gel electrophoresis.

[0066] The NEAR™ reaction including templates for both a *Bacillus subtilis (Bs)* and *Bacillus anthracis (Ba)* DNA was run in the absence of target DNA (negative), in the presence of *Bacillus subtilis* DNA only (27mer product), and in the presence of both *Bacillus subtilis* and *Bacillus anthracis* DNA (27mer and 25mer product respectively) . The target copy number for each genome present in this assay was 500, 000 copies. All reactions contained 500, 000 copies of *Bacillus thuringiensis* as exogenous nucleic acids. Templates varied in concentration between the assays to control the amplification. The assay was run for 10 min at 57 °C, heat denatured at 94 °C for 4 min, and 6 microliters was loaded on to a 20% gel run at 160 V for about 2 hours. The gel was stained with SYBR II fluorescent dye and imaged. The fluorescent bands were quantified and analyzed as the integrated optical density (IOD) .

[0067] **Figure 18.** Gel electrophoresis results for the MS2/Ebola RNA duplex reaction.

[0068] The NEAR™ reaction including templates for both a MS2 and Ebola assay was run in the absence of target RNA (negative, lanes 2-5), in the presence of MS2 only (27mer product, lanes 6 and 7), and in the presence of both MS2 and Ebola RNA (27mer and 25mer product respectively, lanes 8 and 9). The target copy number used in this assay was 1E+6 copies. The assay was run for 10 min at 57 °C. Templates varied in concentration between the assays to control the amplification. Samples were run on a 20% polyacrylamide gel at 160 V for ~2.5 hours. The gel was stained with SYBR II fluorescent dye and imaged. The fluorescent bands were quantified and analyzed as the integrated optical density (IOD).

[0069] **Figure 19.** Mass spec analysis of amplification of DNA from lysed spores.

[0070] Average AUC values from amplified product masses compared for lysed and unlysed samples. Lysed spore samples were then added to master mix and run for 10 minutes at 55 °C, heat denatured for 4 minutes at 94 °C, and run on the mass spec for analysis. AUC values of product peaks were averaged and compared (n = 3).

[0071] **Figure 20.** Demonstration of the capture and extension approach for surface detection.

[0072] A.) Average binding (positive reaction product with no added polymerase), B.) 500, 000 target (positive reaction product with added polymerase), and C.) No target (negative reaction with added polymerase) are compared. The NEAR™ assay was run for 10 minutes at 55 °C, heat denatured at 94 °C for 4 minutes, then added to the plate with capture probe bound to the surface on the 5' end. Polymerase is added to one well of the positive reaction. The plate is incubated at 55 °C for 30 min, washed, SYBR II added, washed 3 times, and read on a Tecan plate reader (495 nm excitation/ 530 nm emission) .

[0073] **Figure 21.** Pseudo-real-time fluorescence detection of the NEAR™ FRET assay with a single template immobilized on a surface in the presence (squares) and absence (open triangles) of 1E+6 copies of genomic DNA.

[0074] Reactions were performed in flat bottom 96-well plates covered with neutravidin. A solution of 1 micromolar FRET-labeled reverse template was incubated with gentle mixing for 1 hr at 37 °C. Wells were washed 3 times with a PBS-Tween solution to release unbound template. NEAR™ reaction mix of the present method was added to the wells (one for each time point taken) and incubated at 58°C on a heating block in a shaking incubator set to 135 RPM. Time points were taken by adding 1 microliter EDTA to the well to stop the reaction. The fluorescence was read from the bottom using a Tecan 100 plate reader.

[0075] **Figure 22.** Limit of Detection Assay for *Chlamydia trachomatis.* A series of assays was performed using 2-fold dilutions of *Chlamydia* target. A) Bar graph of fluorescence detection showing the limit of detection as averaged from 3 assays. B) Bar graph showing the results of individual assays.

[0076] **Figure 23.** Discrimination of *Listeria monocytogenes* from *L. innocua.* Bar graph showing the results of a series of assays that was performed to determine the ability of the assays to discriminate between two different bacteria.

[0077] **Figure 24.** Assay with Viral RNA. Bar graph showing the results of a series of assays of the present methods with various dilutions of a viral RNA target.

[0078] **Figure 25.** Bar graph showing the results of anAssay for etection of the *bar* gene target sequence.

[0079] **Figure 26.** Bar graph showing the results of a assay of the present methods for detection of an miRNA target sequence.

[0080] **Figure 27.** Gc Assay: LOD. A) Bar graph showing the average of a series of assays for detection of a genomic target sequence. B) Results of individual assays, including 50 genomic copies each.

[0081] **Figure 28.** *B. subtilis* NEAR™ assay. A) Standard curve to determine correlation between amount of reference oligonucleotide added to a sample and area under the curve (AUC). B) Bar graph showing the results of assays of the present methods to determine the amount of specific product generated. C) Table showing results of the assay.

[0082] **Figure 29.** Spacer length study. A) Bar graph showing the results of an assay of the present methods to determine the effect of various spacer lengths. B) Template sequences used to obtain different spacer lengths.

[0083] **Figure 30.** Template designs used for the assay shown in Figure 29.

[0084] **Figure 31.** Effect of stabilizing region. A) Graph of the results of assays of the present methods using oligo templates that either include, or don't include, stabilizing regions. B) Expansion of part of the graph of A).

[0085] **Figure 32.** Titration of Mg^{+2} concentration A) Bar graph showing the results of set of NEAR assays using varying amounts of Mg^{+2} . B) Chart describing components of assays.

[0086] **Figure 33.** Drawing depicting mechanisms of the reactions of the present methods.

[0087] **Figure 34.** List of examples of target and oligo template sequences.

Detailed Description

[0088] Provided herein are methods for the exponential amplification of short DNA or RNA sequences.

[0089] Target nucleic acids of the present invention include double-stranded and single-stranded nucleic acid molecules. The nucleic acid may be, for example, DNA or RNA. Where the target nucleic acid is an RNA molecule, the molecule may be, for example, double-stranded, single-stranded, or the RNA molecule may comprise a target sequence that is single-stranded. Where the target nucleic acid is an RNA molecule, the molecule may be double-stranded or single-stranded, or may comprise a target sequence that is single-stranded. Target nucleic acids include, for example, genomic, plasmid, mitochondrial, cellular, and viral nucleic acid. The target nucleic acid may be, for example, genomic, chromosomal, plasmid DNA, a gene, any type of cellular RNA, or a synthetic oligonucleotide. By "genomic nucleic acid" is meant any nucleic acid from any genome, for example, including animal, plant, insect, and bacterial genomes, including, for example, genomes present in spores. Double stranded DNA target nucleic acids include, for example, genomic DNA, plasmid DNA, mitochondrial DNA, viral DNA, and synthetic double stranded DNA or other types of DNA described herein or known in the art. Single-stranded DNA target nucleic acids include, for example, viral DNA, cDNA, and synthetic single-stranded DNA, or other types of DNA described herein or known in the art. RNA target nucleic acids include, for example, messenger RNA, viral RNA, ribosomal RNA, transfer RNA, microRNA and microRNA precursors, and siRNAs or other RNAs described herein or known in the art.

[0090] MicroRNAs, miRNAs, or small temporal RNAs (stRNAs), are short single- stranded RNA sequences, about 21- 23 nucleotides long that are involved in gene regulation. MicroRNAs are thought to interfere with the translation of messenger RNAs as they are partially complementary to messenger RNAs. (see, for example, Ruvkun, G1, Science 294: 797- 99 (2001) ; Lagos- Quintana, M., et al., Science 294: 854- 58 (2001) ; Lau, N.C., et al, Science 294: 858- 62 (2001) ; Lee, R.C., and Ambros, V., Science 294: 862- 64 (2001) ; Baulcombe, D., et al., Science 297: 2002- 03 (2002) ; Llave, C., Science 297: 2053- 56 (2002) ; Hutvagner, G., and Zamore, P.D., Science 297: 2056- 60 (2002)) . MicroRNA may also have a role in the immune system, based on studies recently reported in knockout mice. (see, for example, Wade, N., "Studies Reveal and Immune System Regulator" New York Times, April 27, 2007) . MicroRNA precursors that may also be detected using the methods of the present invention include, for example, the primary transcript (primiRNA) and the pre- miRNA stem- loop- structured RNA that is further processed into miRNA.

[0091] Short interfering RNAs, or siRNAs are at least partially double-stranded, about 20-25 nucleotide long RNA molecules that are found to be involved in RNA interference, for example, in the down-regulation of viral replication or gene expression (see for example Zamore et al., 2000, Cell, 101, 25-33; Bass, 2001, Nature, 411, 428-429; Elbashir et al., 2001, Nature, 411, 494-498; and Kreutzer et al., International PCT Publication No. WO 00/44895; Zernicka-Goetz et al., International PCT Publication No. WO 01/36646; Fire, International PCT Publication No. WO 99/32619; Plaetinck et al., International PCT Publication No. WO 00/01846; Mello and Fire, International PCT Publication No. WO 01/29058; Deschamps-Depaillette, International PCT Publication No. WO 99/07409; and Li et al., International PCT Publication No. WO 00/44914; Allshire, 2002, Science, 297, 1818-1819; Volpe et al., 2002, Science, 297, 1833-1837; Jenuwein, 2002, Science, 297, 2215-2218; and Hall et al., 2002, Science, 297, 2232-2237; Hutvagner and Zamore, 2002, Science, 297, 2056-60; McManus et al., 2002, RNA, 8, 842-850; Reinhart et al., 2002, Gene & Dev., 16, 1616-1626; and Reinhart & Bartel, 2002, Science, 297, 1831).

[0092] The use of the term "target sequence" may refer to either the sense or antisense strand of the sequence, and also refers to the sequences as they exist on target nucleic acids, amplified copies, or amplification products, of the original target sequence. The amplification product may be a larger molecule that comprises the target sequence, as well as at least one other sequence, or other nucleotides. The length of the target sequence, and the guanosine:cytosine (GC) concentration (percent), is dependent on the temperature at which the reaction is run; this temperature is dependent on the stability of the polymerases and nicking enzymes used in the reaction. Those of ordinary skill in the art may run sample assays to determine the appropriate length and GC concentration for the reaction conditions. For example, where the polymerase and nicking enzyme are stable up to 60°C, then the target sequence may be, for example, from 19 to 50 nucleotides in length, or for example, from 20 to 45, 20 to 40, 22 to 35, or 23 to 32 nucleotides in length. The GC concentration under these conditions may be, for example, less than 60%, less than 55%, less than 50%, or less than 45%. The target sequence and nicking enzymes are selected such that the target sequence does not contain nicking sites for any nicking enzymes that will be included in the reaction mix.

[0093] The target sequences may be amplified from many types of samples including, but not limited to samples containing spores, viruses, cells, nucleic acid from prokaryotes or eukaryotes, or any free nucleic acid. For example, the assay can detect the DNA on the outside of spores without the need for lysis. The sample may be isolated from any material suspected of containing the target sequence. For example, for animals, for example, mammals, such as, for example, humans, the sample may comprise blood, bone marrow, mucus, lymph, hard tissues, for example, liver, spleen, kidney, lung, or ovary, biopsies, sputum, saliva, tears, feces, or urine. Or, the target sequence may be present in air, plant, soil, or other materials suspected of containing biological organisms.

[0094] Target sequences may be present in samples that may also contain environmental and contaminants such as dust, pollen, and soot (for example, from diesel exhaust), or clinically relevant matrices such as urine, mucus, or saliva. Target sequences may also be present in waste water, drinking water, air, milk, or other food. Depending on the concentration of these contaminants, sample purification methods known to those of ordinary skill in the art may be required to remove inhibitors for successful amplification. Purification may, for example, involve the use of detergent lysates, sonication, vortexing with glass beads, or a French press. This purification could also result in concentration of the sample target. Samples may also, for be further purified, for example, by filtration, phenol extraction, chromatography, ion exchange, gel electrophoresis, or density dependent centrifugation. In particular embodiments, the sample can be added directly to the reaction mix or pre-diluted and then added to the reaction mix without prior purification of target nucleic acid.

[0095] An oligonucleotide is a molecule comprising two or more deoxyribonucleotides or ribonucleotides, for example, more than three. The length of an oligonucleotide will depend on how it is to be used. The oligonucleotide may be derived synthetically or by cloning.

[0096] The term "complementary" as it refers to two nucleic acid sequences generally refers to the ability of the two sequences to form sufficient hydrogen bonding between the two nucleic acids to stabilize a double-stranded nucleotide sequence formed by hybridization of the two nucleic acids. In the two sequences, all nucleotides in one sequence may be complementary to counterpart nucleotides in the other sequence. In some embodiments, there may be a few mismatches between counterpart nucleotides in the two sequences (i.e., non-complementary nucleotides), such as 1 mismatch in 10 nucleotides, 1 mismatch in 20 nucleotides, or 1 mismatch in 30 nucleotides, for example, which sequences are referred to as "substantially complementary" herein. As shown in Figures 1A-1D, each template nucleic acid often includes a recognition region complementary to, or substantially complementary to, a target nucleic acid strand (or complement thereof) to which the template nucleic acid hybridizes. Also shown in Figures 1A-1D, each template nucleic acid often includes a stabilizing region 5' of the recognition region and nick agent recognition region that is not complementary or substantially complementary to the target nucleic acid sequence or complement thereof.

[0097] As used herein, "hybridization" and "binding" are used interchangeably and refer to the non-covalent binding or "base pairing" of complementary nucleic acid sequences to one another. Whether or not a particular probe remains base paired with a polynucleotide sequence depends on the degree of complementarity, the length of the probe, and the stringency of the binding conditions. The higher the stringency, the higher must be the degree of complementarity, and/or the longer the probe for binding or base pairing to remain stable.

[0098] As used herein, "stringency" refers to the combination of conditions to which nucleic acids are subjected that cause double-stranded nucleic acid to dissociate into component single strands such as pH extremes, high temperature, and salt concentration. The phrase "high stringency" refers to hybridization conditions that are sufficiently stringent or restrictive such that only specific base pairings will occur. The specificity should be sufficient to allow for the detection of unique sequences using an oligonucleotide probe or closely related sequence under standard Southern hybridization protocols (as described in J. Mol. Biol. 98:503 (1975)).

[0099] Templates are defined as oligonucleotides that bind to a recognition region of a target sequence and also contain a nicking enzyme binding region upstream of the recognition region and a stabilizing region upstream to the nicking enzyme binding region.

[0100] By "recognition region" is meant a nucleic acid sequence on the template that is complementary or substantially complementary to a nucleic acid sequence on the target sequence. By "recognition region on the target sequence" is meant the nucleotide sequence on the target sequence that is complementary or substantially complementary to, and binds to, the template.

[0101] By "stabilizing region" is meant a nucleic acid sequence having, for example, about 50% GC content, designed to stabilize the molecule for, for example, the nicking and/or extension reactions.

[0102] In describing the positioning of certain sequences on nucleic acid molecules, such as, for example, in the target sequence, or the template, it is understood by those of ordinary skill in the art that the terms "3' " and "5' " refer to a location of a particular sequence or region in relation to another. Thus, when a sequence or a region is 3' to or 3' of another sequence or region, the location is between that sequence or region and the 3' hydroxyl of that strand of nucleic acid. When a location in a nucleic acid is 5' to or 5' of another sequence or region, that means that the location is between that sequence or region and the 5' phosphate of that strand of nucleic acid.

[0103] The polymerase is a protein able to catalyze the specific incorporation of nucleotides to extend a 3' hydroxyl

terminus of a primer molecule, such as, for example, the template oligonucleotide, against a nucleic acid target sequence. The polymerase may be, for example, thermophilic so that it is active at an elevated reaction temperature. It may also, for example, have strand displacement capabilities. It does not, however, need to be very processive (30-40 nucleotides for a single synthesis are sufficient). Often, the polymerase used does not have 5'-3' exonuclease activity. If the polymerase also has reverse transcriptase activity (such as Bst (large fragment), 9°N, Therminator, Therminator II, etc.) the reaction can also amplify RNA targets in a single step without the use of a separate reverse transcriptase. More than one polymerase may be included in the reaction, in one example one of the polymerases may have reverse transcriptase activity and the other polymerase may lack reverse transcriptase activity. In exemplary embodiments, the polymerase is BST (large fragment). The polymerase may be selected from, for example, the group consisting of one or more of the polymerases listed in Table 1.

Table 1

Polymerase
Bst DNA polymerase
Bst DNA polymerase (Large fragment)
9°Nm DNA polymerase
Phi29 DNA polymerase
DNA polymerase I (*E.coli*)
DNA polymerase I, Large (Klenow) fragment
Klenow fragment (3'-5' exo-)
T4 DNA polymerase
T7 DNA polymerase
Deep Vent$_R$™ (exo-) DNA Polymerase
Deep Vent$_R$™ DNA Polymerase
DyNAzyme™ EXT DNA
DyNAzyme™ II Hot Start DNA Polymerase
Phusion™ High-Fidelity DNA Polymerase
Therminator™ DNA Polymerase
Therminator™ II DNA Polymerase
Vent$_R$® DNA Polymerase
Vent$_R$® (exo-) DNA Polymerase
RepliPHI™ Phi29 DNA Polymerase
rBst DNA Polymerase
rBst DNA Polymerase, Large Fragment (IsoTherm™ DNA Polymerase)
MasterAmp™ AmpliTherm™ DNA Polymerase
Taq DNA polymerase
Tth DNA polymerase
Tfl DNA polymerase
Tgo DNA polymerase
SP6 DNA polymerase
Tbr DNA polymerase
DNA polymerase Beta
ThermoPhi DNA polymerase

(continued)

Polymerase
Pyrophage 3173 (Lucigen)

5

[0104] The following non- limiting examples of Reverse Transcriptases (RT) can be used in the reactions of the present method to improve performance when detecting an RNA sequence: OmniScript (Qiagen), SensiScript (Qiagen), MonsterScript (Epicentre), Transcriptor (Roche), HIV RT (Ambion), SuperScript III (Invitrogen), ThermoScript (Invitrogen), Thermo- X (Invitrogen), ImProm II (Promega) .

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[0105] These different RTs perform at different levels in the standard reaction buffer, and this performance rating is listed below. A "+" indicates that the amplification reaction results in specific product. More "+"s indicate that the reaction works better, with "+++++" indicating excellent results. A "-" indicates that the reaction did not result in specific product, or did not result in specific product over background.

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Table 2

OmniScript** (Qiagen)	+++++
SensiScript (Qiagen)	+++
MonsterScript (Epicentre)	+++
Transcriptor (Roche)	++
HIV RT* (Ambion)	+
SuperScript III (Invitrogen)	-
ThermoScript (Invitrogen)	-
Thermo-X (Invitrogen)	-
ImProm II (Promega)	-

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[0106] "Nicking" refers to the cleavage of only one strand of the double-stranded portion of a fully or partially double-stranded nucleic acid. The position where the nucleic acid is nicked is referred to as the nicking site or nicking site. The recognition sequence that the nicking enzyme recognizes is referred to as the nicking enzyme binding site. "Capable of nicking" refers to an enzymatic capability of a nicking enzyme.

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[0107] The nicking enzyme is a protein that binds to double-stranded DNA and cleaves one strand of a double-stranded duplex. The nicking enzyme may cleave either upstream or downstream of the binding site, or nicking enzyme recognition site. In exemplary embodiments, the reaction comprises the use of nicking enzymes that cleave or nick downstream of the binding site (top strand nicking enzymes) so that the product sequence does not contain the nicking site. Using an enzyme that cleaves downstream of the binding site allows the polymerase to more easily extend without having to displace the nicking enzyme. The nicking enzyme must be functional in the same reaction conditions as the polymerase, so optimization between the two ideal conditions for both is necessary. Nicking enzymes are available from, for example, New England Biolabs (NEB) and Fermentas. The nicking enzyme may, for example, be selected from the group consisting of one or more of the nicking enzymes listed in Table 3.

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Table 3

Nicking Enzyme	Alternate Name
Nb.BbvCI	
Nb.Bpu10I	
Nb.BsaI	
Nb.BsmI	
Nb.BsrDI	
Nb.BstNBIP	
Nb.BstSEIP	
Nb.BtsI	

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(continued)

Nicking Enzyme	Alternate Name
Nb.SapI	
Nt.AlwI	
Nt.BbvCI	
Nt.BhaIIIP	
Nt.Bpu10I	
Nt.Bpu10IB	
Nt.BsaI	
Nt.BsmAI	
Nt.BsmBI	
Nt.BspD61	
Nt.BspQI	
Nt.Bst91	
Nt.BstNBI	N.BstNB I
Nt.BstSEI	
Nt.CviARORFMP	
Nt.CviFRORFAP	
Nt.CviPII	Nt.CviPIIm
Nt.CviQII	
Nt.CviQXI	
Nt.EsaSS1198P	
Nt.MlyI	
Nt. SapI	

[0108] Nicking enzymes may be, for example, selected from the group consisting of Nt.BspQI (NEB), Nb.BbvCI (NEB), Nb.BsmI (NEB), Nb.BsrDI (NEB), Nb.BtsI (NEB), Nt.AlwI (NEB), Nt.BbvCI (NEB), Nt.BstNBI (NEB), Nt.CviPII (NEB), Nb.Bpu101 (Fermantas), and Nt.Bpu101 (Fermentas) . In certain embodiments, the nicking enzyme is selected from the group consisting of Nt.NBst.NBI, Nb.BsmI, and Nb.BsrDI. Those of ordinary skill in the art are aware that various nicking enzymes other than those mentioned specifically herein may be used in the present methods.

[0109] Nicking enzymes and polymerases of the present methods may be, for example, stable at room temperature, the enzymes may also, for example, be stable at temperatures up to 37°C , 42°C , 60°C, 65°C, 70°C, 75°C, 80°C, or 85°C. In certain embodiments, the enzymes are stable up to 60°C.

[0110] An enzyme is "thermophilic" when it is stable at temperatures up to 37°C , 42°C , 50-60C, 54-60°C, 56-58°C, 60°C, 65°C, 70°C, 75°C, 80°C, or 85°C

[0111] Product or amplified product is defined as the end result of the extension of the template along the target that is nicked and released. This product can then feed back into the amplification cycle, or it can anneal to its complement or a molecular beacon.

[0112] A "native nucleotide" refers to adenylic acid, guanylic acid, cytidylic acid, thymidylic acid, or uridylic acid. A "derivatized nucleotide" is a nucleotide other than a native nucleotide.

[0113] The reaction may be conducted in the presence of native nucleotides, such as, for example, dideoxyribonucleoside triphosphates (dNTPs) . The reaction may also be carried out in the presence of labeled dNTPs, such as, for example, radiolabels such as, for example, ^{32}P, ^{33}P, ^{125}I, or ^{35}S, enzyme labels such as alkaline phosphatase, fluorescent labels such as fluorescein isothiocyanate (FITC), biotin, avidin, digoxigenin, antigens, haptens, or fluorochromes. These derivatized nucleotides may, optionally, be present in the templates.

[0114] By "constant temperature," "isothermal conditions," "essentially isothermal," or "isothermally" is meant a set of reaction conditions where the temperature of the reaction is kept essentially or substantially constant during the course

of the amplification reaction. An advantage of the amplification method of the present methods is that the temperature does not need to be cycled between an upper temperature and a lower temperature. The nicking and the extension reaction will work at the same temperature or within the same narrow temperature range. However, it is not necessary that the temperature be maintained at precisely one temperature. If the equipment used to maintain an elevated temperature allows the temperature of the reaction mixture to vary by a few degrees, or few tenths of a degree, such as, for example, less than 1 degree, 0.8 degrees, 0.6 degrees, 0.4 degrees, or 0.2 degrees, this is not detrimental to the amplification reaction, and may still be considered to be an isothermal reaction.

[0115] The term "multiplex amplification" refers to the amplification of more than one nucleic acid of interest. For example, it can refer to the amplification of multiple sequences from the same sample or the amplification of one of several sequences in a sample as discussed, for example, in U.S. Patent Nos. 5,422,252; and 5,470,723, which provide examples of multiplex strand displacement amplification. The term also refers to the amplification of one or more sequences present in multiple samples either simultaneously or in step-wise fashion.

Template Design

[0116] Forward and Reverse templates, and first and second templates, are designed so that there is a stabilizing region at the 5' end, a nicking enzyme binding site and a nicking site downstream of the stabilizing region, and a recognition region downstream of the nicking enzyme binding site and the nicking site on the 3' end of the oligonucleotide. The total oligo length can range from 19 to 40, for example from 19-40, 23-40, 20-30, 20-24, 23-24, 23-32, 25-40, 27-40, or 27-35 nucleotides depending on the length of each individual region, the temperature, the length of the target sequence, and the GC concentration. One of ordinary skill in the art would know how to balance these features of the templates. The templates may be designed so that they, together, would bind to less than or equal to 100% of the target sequence, one binding to the sense strand, and one to the antisense strand. The length of each template does not need to be the same length as the other template. For example, where the forward template binds to about 60% of the target antisense strand, the reverse template may, for example, bind to about 40% of the target sense strand. The templates may be designed to allow for spacer bases on the target sequence, that do not bind to either template. The templates thus may be designed to bind to about 30%, about 40%, about 50%, or about 60% of the target sequence.

[0117] The recognition region of the forward template is designed to be substantially identical or identical to the 5' region of the target sense strand and complementary or substantially complementary to the 3' end of the target site antisense strand. The recognition region of the forward template is of any suitable length, for example, about 8, 9, 10, 11, 12, 13, 14, 15 or 16 bases in length, and sometimes 8-16, 9-16, 10-16, 8-12, 8-15, 9-15, 10-15, or 11-14 nucleotides long. In exemplary embodiments, the length is 11-13, 11-12, 12, or 12-13 nucleotides long. The recognition region of the reverse template is designed to be substantially complementary or complementary to the 3' end of the target site sense strand. The recognition region of the reverse template is of any suitable length, for example, about 8, 9, 10, 11, 12, 13, 14, 15 or 16 bases in length, and sometimes 8-16, 9-16, 10-16, 8-12, 8-15, 9-15, 10-15, or 11-14 nucleotides long. In exemplary embodiments, the length is 11-13, 11-12, 12, or 12-13 nucleotides long. The length of the recognition region of the first template may either be the same as the length of the recognition region of the second template, or may be different.

[0118] A recognition sequence of a template often is complementary or substantially complementary to a unique sequence, or substantially unique sequence, of an organism. The term "unique sequence" as used herein refers to a nucleotide sequence in an organism that is present in no other known organism. A "substantially unique sequence" as used herein refers to a nucleotide sequence present in a specific family of organisms, or in up to only about 1, 2, 3, 4, 5, 6, 7, 8, 9, or 10 other organisms. In some embodiments, a unique sequence or substantially unique sequence is present in ribosomal RNA or in the sense or antisense strand of DNA encoding ribosomal RNA.

[0119] Those of ordinary skill in the art are able to determine the appropriate recognition region length for optimal, efficient, amplification. In certain embodiments, to provide appropriate specificity, an 8 base-length template recognition region is a lower limit. The analytical specificity of the reaction is linked to the sum of the recognition regions of the two templates, the forward and the reverse template. If each template has a recognition region of 8 nucleotides, for example, that confers an assay that is able to detect a unique combination of 8+8=16 nucleotides, referred to as the "target size." For a given DNA strand, a target size of 16 nucleotides has 4.29x109 possible combinations. The human genome is 3.3x109 nucleotides long. Therefore, statistically, a specific 16 nucleotide sequence is expected to occur approximately once in the human genome. As the target size decreases, for example to 15 nucleotides, that would be expected to occur, on average, 3 times in the human genome (1.07x109 possibilities in 3.3x109 occurrences), and would therefore not be as specific as a 16 nucleotide target size. For an assay with a recognition region of 7 nucleotides, conferring an assay target size of 14 bases, this would be expected to be present in the human genome 12 times (2.68x108 possibilities in 3.3x109 occurrences). This would generate an assay with reduced specificity that would have less value in a diagnostic setting. Therefore, an 8 base recognition region for each template is often considered to be the lower limit for certain assays.

Table 4

Assay Target Size	# unique possibilities
N	4^N
14	2.68E+08
15	1.07E+09
16	4.29E+09
17	1.72E+10
18	6.87E+10
19	2.75E+11
20	1.10E+12
21	4.40E+12
22	1.76E+13
23	7.04E+13
24	2.81E+14
25	1.13E+15
26	4.50E+15

[0120] Amplification ssays in accordance with the present invention were conducted to determine the optimal length of the recognition region. In 10 minute assays, using either 0 or 100,000 copies of target DNA, a 20 mer recognition region template set did not produce detectable specific product, while specific product was detected using a 12 mer recognition region template set. The use of a 16 mer recognition region template set resulted in specific detectable product, but four-fold less specific product was detected than in an assay using the 12 mer recognition region template set. In certain embodiments, the use of a 15 mer recognition region template set generated more specific product than a 16 mer recognition region template set.

[0121] Thus, in certain exemplary embodiments, methods are provided for amplifying a double stranded nucleic acid target sequence comprising contacting a target DNA molecule comprising a double- stranded target sequence, having a sense strand and an antisense strand, with a forward template and a reverse template, wherein the forward template comprises a nucleic acid sequence comprising a recognition region at the 3' end that is complementary or substantially complementary to the 3' end of the target sequence antisense strand, wherein the recognition region is from 8 to 15 nucleotides in length; a nicking enzyme binding site and a nicking site upstream of the recognition region and a stabilizing region upstream of the nicking enzyme binding site and the nicking site; the reverse template comprises a nucleotide sequence comprising a recognition region at the 3' end that is complementary or substantially complementary to the 3' end of the target sequence sense strand, wherein the recognition region is from 8 to 15 nucleotides in length, a nicking enzyme binding site and a nicking site upstream of the recognition region, and a stabilizing region upstream of the nicking enzyme binding site and the nicking site; providing a first nicking enzyme that is capable of nicking at the nicking site of the forward template, and does not nick within the target sequence; providing a second nicking enzyme that is capable of nicking at the nicking site of the reverse template and does not nick within the target sequence; and providing a DNA polymerase; under conditions wherein amplification is performed by multiple cycles of the polymerase extending the forward and reverse templates along the target sequence producing a double- stranded nicking site, and the nicking enzymes nicking at the nicking sites, producing an amplification product. Thus, in certain embodiments, the recognition region of the forward or reverse template, or each of the forward and reverse templates is 8, 9, 10, 11, 12, 13, 14, or 15 nucleotides in length. In certain embodiments, the target sequence comprises from 1 to 5 nucleotides more than the sum of the nucleotides of the forward template recognition region and the reverse template recognition region

[0122] In another exemplary embodiment, methods are provided for amplifying a single-stranded nucleic acid target sequence, comprising contacting a target nucleic acid comprising a single-stranded target sequence with a reverse template, wherein the reverse template comprises a nucleotide sequence comprising a recognition region at the 3' end that is complementary or substantially complementary to the 3' end of the target sequence wherein the recognition region is from 8 to 15 nucleotides in length, a nicking enzyme binding site and a nicking site upstream of the recognition region, and a stabilizing region upstream of the nicking enzyme binding site and the nicking site; providing a first nicking enzyme that is capable of nicking at the nicking site of the reverse template, and does not nick within the target sequence; providing a DNA polymerase under conditions wherein the polymerase extends the reverse template along the target sequence; contacting the extended reverse template with a forward template, wherein the forward template comprises a recognition region at the 3'end that is identical to the 5'end of the target sequence wherein the recognition region is from 8 to 15 nucleotides in length, a nicking enzyme binding site and a nicking site upstream of the recognition region,

and a stabilizing region upstream of the nicking enzyme binding site and the nicking site; providing a second nicking enzyme that is capable of nicking at the nicking site of the forward template and does not nick within the target sequence; under conditions wherein amplification is performed by multiple cycles of the polymerase extending the forward and reverse templates along the target sequence producing a double-stranded nicking site, and the nicking enzymes nicking at the nicking sites, producing an amplification. Thus, in certain embodiments, the recognition region of the forward or reverse template, or each of the forward and reverse templates, is 8, 9, 10, 11, 12, 13, 14, or 15 nucleotides in length. In certain embodiments, the target sequence comprises from 1 to 5 nucleotides more than the sum of the nucleotides of the forward template recognition region and the reverse template recognition region

[0123] In certain embodiments, the temperature at which the amplification reaction is conducted is lower than the melting temperature (T_m) of a template and target. In certain embodiments, the reaction temperature is, for example, from 1 °C -10 °C, 1 °C -8 °C, 1 °C - 6 °C, 1 °C -4 °C, 1 °C -2 °C, 2 °C -10 °C, 2 °C -8 °C, 2 °C -6 °C, 2 °C -4 °C, 2 °C -2 °C from 2 °C-4 °C or from 2 °C, 3 °C, 4 °C, 5 °C, 6°C, 7°C, or 8°C less than the T_m of a template and target. The reaction temperature also often is lower than the T_m of the reaction products (e.g., products of nicking and polymerase extension of the amplification duplex shown in Figure 1B and Figure 1C after step 9A). The reaction temperature may be higher than the T_m of the initial template/target sequence complex (drawing above step (1) of Figure 1A). Once the template is extended to form a stable complex, the T_m of the stable complex is higher than the reaction temperature.

[0124] Thus, the T_m of a template/ target nucleic acid target often is higher than the reaction temperature, and sometimes the T_m is 5 °C or more higher than the reaction temperature, or for example, about 1 °C, 2 °C, 3 °C, 4 °C, 5 °C, 6 °C, 7 °C, or 8 °C or more higher than the reaction temperature. The T_m of each portion of the nicked strand after nicking often is higher than the reaction temperature, and sometimes the T_m of each nicked portion is 5 °C or more higher than the reaction temperature, or for example, about 1 °C, 2 °C, 3 °C, 4 °C, 5 °C, 6 °C, 7 °C, or 8 °C or more higher than the reaction temperature. The T_m of the template and target may be calculated, for example, using the program provided for the IDT Oligo Analyzer (Integrated DNA Technologies) at World Wide Web URL idtdna.com/ analyzer/ Applications/ OligoAnalyzer/ considering the salt concentration of the reaction conditions. As discussed at the IDT website, the T_m calculations using the Analyzer are conducted as follows:

[0125] Melting temperature (T_M) is the temperature at which an oligonucleotide duplex is 50% in single- stranded form and 50% in double- stranded form. The Oligo Analyzer estimates T_M from the nearest- neighbor two- state model, which is applicable to short DNA duplexes,

$$T_M(^\circ C) = \frac{\Delta H^\circ}{\Delta S^\circ + R \ln[\text{oligo}]} - 273.15$$

where ΔH° (enthalpy) and ΔS° (entropy) are the melting parameters calculated from the sequence and the published nearest neighbor thermodynamic parameters, R is the ideal gas constant (1.987 $cal \cdot K^{-1} mole^{-1}$), [oligo] is the molar concentration of an oligonucleotide, and the constant of- 273.15 converts temperature from Kelvin to degrees of Celsius. The most accurate, nearest- neighbor parameters were obtained from the following publications for DNA/DNA base pairs (Allawi, H., SantaLucia, J., Jr., Biochemistry, 36, 10581), RNA/DNA base pairs (Sugimoto, N. et al., Biochemistry, 34, 11211), RNA/RNA base pairs (Xia, T. et al., Biochemistry, 37, 14719), and LNA/DNA base pairs (McTigue, P.M. et al., Biochemistry, 43, 5388) .

[0126] T_M depends on monovalent salt concentration ($[Na^+]$) of the solvent. The linear T_M correction is a method known in the art. As discussed in the IDT website, scientists at IDT performed a large set of UV melting experiments (~3000 measurements) on about 100 short DNA duplexes in a variety of sodium buffers and determined that this linear function is inaccurate. OligoAnalyzer employs the improved quadratic T_M salt correction function (Owczarzy, R. et al., Biochemistry, 43, 3537),

$$\frac{1}{T_M(Na^+)} = \frac{1}{T_M(1M\ Na^+)} + \left(4.29 f(GC) - 3.95\right) \times 10^{-5} \ln[Na^+] + 9.40 \times 10^{-6} \ln^2[Na^+]$$

where f (GC) is the fraction of GC base pairs.

[0127] In certain embodiments, the lengths of the recognition regions are adjusted so that there is at least one nucleotide in the target sequence that is not in the forward template's recognition region and also does not have its complement in the reverse template's recognition region. These spacer bases (which form the "spacer region") are nucleotides contained within the target sequence that lie in between the 3' ends of the forward and reverse templates. The spacer bases are shown in, for example, Figure 30, where they are indicated as the section of the target sense and antisense sequences between the 3' ends of the forward and reverse templates, also indicated within the "spacer region." For

example, when templates T2 and T1 of Figure 30 are used with the target, the target sense strand has 1 spacer base (or, a gap of 1 nucleotide)- T, and the target antisense strand has 1 spacer base (or, a gap of 1- nucleotide)- A. In certain embodiments, 5 spacer bases or less are present in the target sequence. In exemplary embodiments, the number of spacer bases is 2 to 3. In certain embodiments, the number of spacer bases is 1, 2, or 3. In other embodiments, there is 1 spacer base. In other embodiments, there are 2 spacer bases. In other embodiments, there are 3 spacer bases. In other embodiments, the number of spacer bases is 1, 2, 3, 4, or 5.

[0128] Thus, in exemplary embodiments of the present methods, the target sequence comprises from 1 to 5 nucleotides between the target sequence nucleotide that hybridizes to the 3' end of the first template and the corresponding nucleotide to the nucleotide of the complement of the first strand that hybridizes to the 3' end of the second template. By "corresponding nucleotide" is meant the nucleotide on one strand of the target nucleotide sequence that hybridizes to the complementary strand of the target nucleotide sequence when the two strands are aligned. These 1 to 5 nucleotides are also called spacer bases.

[0129] These spacer bases allow for distinction of the true amplified product from any background products amplified by extension due to overlapping templates in a similar manner to primer-dimers. This consideration allows for improved discrimination between background noise and amplification of a target sequence. However, these spacer bases are not required for the amplification to proceed.

[0130] Thus, in certain exemplary embodiments, methods are provided for amplifying a double stranded nucleic acid target sequence comprising contacting a target DNA molecule comprising a double- stranded target sequence, having a sense strand and an antisense strand, with a forward template and a reverse template, wherein the forward template comprises a nucleic acid sequence comprising a recognition region at the 3' end that is complementary or substantially complementary to the 3' end of the target sequence antisense strand; a nicking enzyme binding site and a nicking enzyme binding site and a nicking site upstream of the recognition region and a stabilizing region upstream of the nicking enzyme binding site and the nicking site; the reverse template comprises a nucleotide sequence comprising a recognition region at the 3' end that is complementary or substantially complementary to the 3' end of the target sequence sense strand, a nicking enzyme binding site and a nicking site upstream of the recognition region, and a stabilizing region upstream of the nicking enzyme binding site and the nicking site; providing a first nicking enzyme that is capable of nicking at the nicking site of the forward template, and does not nick within the target sequence; providing a second nicking enzyme that is capable of nicking at the nicking site of the reverse template and does not nick within the target sequence; and providing a DNA polymerase; under conditions wherein amplification is performed by multiple cycles of the polymerase extending the forward and reverse templates along the target sequence producing a double- stranded nicking site, and the nicking enzymes nicking at the nicking sites, producing an amplification product, wherein the target sequence comprises from 1 to 5 nucleotides more than the sum of the nucleotides of the forward template recognition region and the reverse template recognition region. Thus, in certain embodiments, the target sequence comprises 1, 2, 3, 4, or 5 nucleotides more than the sum of the nucleotides of the forward template recognition region and the reverse template recognition region.

[0131] In another exemplary embodiment, methods are provided for amplifying a single-stranded nucleic acid target sequence, comprising contacting a target nucleic acid comprising a single-stranded target sequence with a reverse template, wherein the reverse template comprises a nucleotide sequence comprising a recognition region at the 3' end that is complementary or substantially complementary to the 3' end of the target sequence, a nicking enzyme binding site and a nicking site upstream of the recognition region, and a stabilizing region upstream of the nicking enzyme binding site and the nicking site; providing a first nicking enzyme that is capable of nicking at the nicking site of the reverse template, and does not nick within the target sequence; providing a DNA polymerase under conditions wherein the polymerase extends the reverse template along the target sequence; contacting the extended reverse template with a forward template, wherein the forward template comprises a recognition region at the 3'end that is identical to the 5'end of the target sequence a nicking enzyme binding site and a nicking site upstream of the recognition region, and a stabilizing region upstream of the nicking enzyme binding site and the nicking site; providing a second nicking enzyme that is capable of nicking at the nicking site of the forward template and does not nick within the target sequence; under conditions wherein amplification is performed by multiple cycles of the polymerase extending the forward and reverse templates along the target sequence producing a double-stranded nicking site, and the nicking enzymes nicking at the nicking sites, producing an amplification product , wherein the target sequence comprises from 1 to 5 nucleotides more than the sum of the nucleotides of the forward template recognition region and the reverse template recognition region. Thus, in certain embodiments, the target sequence comprises 1, 2, 3, 4, or 5 nucleotides more than the sum of the nucleotides of the forward template recognition region and the reverse template recognition region.

[0132] The nicking enzyme binding site sequence of the template depends on which nicking enzyme is chosen for each template. Different nicking enzymes may be used in a single assay, but a simple amplification may, for example, employ a single nicking enzyme for use with both templates. Thus, the embodiments of the present methods include those where both templates comprise recognition sites for the same nicking enzyme, and only one nicking enzyme is used in the reaction. In these embodiments, both the first and second nicking enzymes are the same. The present

methodalso includes those embodiments where each template comprises a recognition site for a different nicking enzyme, and two nicking enzymes are used in the reaction.

[0133] For example, in the case of Nt.BstNBI, the enzyme binding site is 5'-GAGTC-3' and the enzyme nicks the top strand four nucleotides down stream of this site (i.e., GAGTCNNNN^). The amplification reaction shows little dependence on the sequence of these four nucleotides (N), though optimal sequence of this region is 25% or less GC content and with a thymine adjacent to the 5' nucleotide of the binding region. The latter stipulation allows for the priming ability of products that have an additional adenine added on by the polymerase. The sequence of the four nucleotides can be optimized to create or eliminate the presence of hairpins, self-dimers, or heterodimers, depending on the application.

[0134] The stabilizing region on the 5' end of the template oligonucleotide is designed to be roughly 50% GC. Thus, the GC content may be, for example, about 40%-60%, about 42%-58%, about 44%-56%, about 46%-54%, about 48%-52%, or about 49%-51%. These parameters result in a stabilizing region length of 8-11 nucleotides for the Nt.BstNBI enzyme, though lengths as short as 6 and as long as 15 nucleotides have been tested and were shown to work in this amplification method. Longer stabilizing regions or increased %GC to greater than 50 % could further stabilize the nicking and extension reactions at higher reaction temperatures. The sequence of the 5' stabilizing regions of forward and reverse templates are usually identical, but can be varied if the aim is to capture each product strand independently. The sequence of this region should not interfere with the nicking site or the recognition region, though short internal hairpins within the template sequence have been shown to have improved real-time results.

[0135] In certain embodiments, one or more agents that destabilize nucleic acid interaction (e.g., inter- strand or intra-strand interactions) are included in an amplification process, and in alternative embodiments, one or more of such agents are not included in an amplification process. Examples of agents that destabilize nucleic acid interaction are those that destabilize double- stranded structure (e.g., double- stranded DNA), and/or structures within a strand (e.g., secondary or tertiary structures in RNA), and include, without limitation, betaines and other tetra- ammonium compounds, formamide, glycerol, sorbitol, sodium perchlorate, dimethylsulfoxide (DMSO), lower alkyl alcohols (e.g., ethanol; 1- 4 carbon alcohols), urea, trialkyl ammonium salts (e.g., triethyl ammonium chloride), single strand binding (ssb) proteins, such as, for example, E. coli ssb, helicases, such as, for example, E. coli DNA helicases I, II, or IV, lower alkyl (1- 4 C) alcohols, and the like. Without being bound by theory, such agents lower the melting temperature (T_m) of nucleic acid interactions (e.g., lower duplex T_m) . Those of ordinary skill in the art may determine the appropriate destabilizing agent and appropriate destabilizing agent concentration for the reaction, considering, for example, the amount of destabilization as well as the need to maintain enzymatic activity. Examples of concentrations include about 10% glycerol, about 10% sodium perchlorate, about 10% DMSO, about 10% sorbitol, about 2.4 molar triethyl ammonium chloride, and about greater than 1, 2, 3, 4, or 5 molar betaine, for example, about 5- 6 molar betaine. Betaine, or N, N, N- trimethylglycine, may be purchased from, for example Sigma- Aldrich, for example, catalog numbers B2629 or B0300. It may be used, for example, in combination with low concentrations of DMSO, for example, about 1- 2, or about 1.3% DMSO to about 1M betaine.

[0136] The templates of the present methods may include, for example, spacers, blocking groups, and modified nucleotides. Modified nucleotides are nucleotides or nucleotide triphosphates that differ in composition and/or structure from natural nucleotide and nucleotide triphosphates. Modified nucleotide or nucleotide triphosphates used herein may, for example, be modified in such a way that, when the modifications are present on one strand of a double-stranded nucleic acid where there is a restriction endonuclease recognition site, the modified nucleotide or nucleotide triphosphates protect the modified strand against cleavage by restriction enzymes. Thus, the presence of the modified nucleotides or nucleotide triphosphates encourages the nicking rather than the cleavage of the double-stranded nucleic acid. Blocking groups are chemical moieties that can be added to the template to inhibit target sequence-independent nucleic acid polymerization by the polymerase. Blocking groups are usually located at the 3' end of the template. Examples of blocking groups include, for example, alkyl groups, non-nucleotide linkers, phosphorothioate, alkane-diol residues, peptide nucleic acid, and nucleotide derivatives lacking a 3'-OH, including, for example, cordycepin. Examples of spacers, include, for example, C3 spacers. Spacers may be used, for example, within the template, and also, for example, at the 5' end, to attach other groups, such as, for example, labels.

[0137] Unmodified nucleotides often are provided for template extension. Unmodified nucleotides and nucleotide derivatives often are not provided for incorporation into extended templates. In certain embodiments, however, one or more modified nucleotides or nucleotide derivatives may be provided and incorporated into an extended template.

[0138] The amplification reaction may also include helper oligonucleotides. Helper oligonucleotides are oligonucleotides that are, for example, about 5-10, 5-15, 5-20, nucleotides long. The presence of helper oligonucleotides may increase the speed, amount, or sensitivity of the amplification reaction. Helper oligonucleotides are not incorporated into the final product. Those of ordinary skill in the art would be able to determine the appropriate helper oligonucleotides to add to a reaction, as well as the amount to add. One example of a way to determine the appropriate helper oligonucleotides is to synthesize oligonucleotides that are complementary to various regions of the target nucleic acid or its complement, and add them to the assay in varying amounts, comparing the assay with the helper oligonucleotides to one without helper oligonucleotides as a control. Helper oligonucleotides may be synthesized that are complementary to regions upstream or downstream of the recognition region, or its complement. For example, sets of helper oligonucleotides about

10 bases long may be synthesized that are complementary to regions spaced every 5-10 bases upstream or downstream of the recognition region, then tested in pairs for their ability to enhance the amplification reaction.

Detailed Mechanism of Amplification

[0139] Amplification reactions of the present methods require the presence of a nucleic acid target, at least two template oligonucleotides, a nicking enzyme, for example, a thermophilic nicking enzyme, a thermophilic polymerase, and buffer components all held at the reaction temperature. The recognition region of the templates interacts with the complementary or substantially complementary target sequence. Since the melting temperature of the complementary or substantially complementary regions of the target and template is well below the reaction temperature, the interaction between the two nucleic acid strands is transient, but allows enough time for a thermophilic polymerase to extend from the 3' end of the template along the target strand. Experiments have shown that certain polymerases bind to single-stranded oligonucleotides. The pre-formation of this complex can facilitate the speed of the amplification process.

[0140] For a double-stranded target, both templates can interact with the corresponding target strands simultaneously (forward template with the antisense strand and reverse template with the sense strand) during the normal breathing of double-stranded DNA. The target may also be generated by a single or double nick sites within the genome sequence. For a single-stranded target (either RNA or DNA), the reverse template binds and extends first (Figure 1, Step 1 and 2). The extended sequence contains the complement to the forward template. The forward template then displaces a region of the target and binds to the 3' synthesized region complementary or substantially complementary to the recognition region of the forward template (Step 3). Alternatively, another reverse template can also displace the initial extended reverse template at the recognition region to create a single-stranded extended reverse template for the forward template to bind. The initial binding and extension of the templates is facilitated by a non-processive polymerase that extends shorter strands of DNA so that the melting temperature of the synthesized product is above the reaction temperature. The single-stranded product is then available for the next template recognition site to bind and polymerase to extend.

[0141] The forward template is extended to the 5' end of the reverse template, creating a double-stranded nicking enzyme binding site for the reverse template (Step 5). The nicking enzyme then binds to the duplex and nicks directly upstream of the recognition sequence of the reverse template strand (in the case of a top-strand nicking enzyme) (Step 6). The nucleic acid sequence downstream of the nick is either released (if the melting temperature is near the reaction temperature) and/or is displaced by the polymerase synthesis from the 3'-OH nick site. Polymerase extends along the forward template to the 5' end of the forward template (Step 8). The double-strand formed from the extension of both templates creates a nicking enzyme binding site on either end of the duplex. This double-strand is termed the NEAR™ amplification duplex. When nicking enzyme binds and nicks, either the target product located in between the two nick sites (with 5'-phosphate and 3'-OH) is released, usually ranging in length from (but is not limited to) 23 to 29 bases (Steps 9-11A), or the singly-nicked product containing the target product and the reverse complement of the nick site and stability region of the template (usually 36 to 48 bases in length) is released (Steps 9-11B). Another depiction of mechanisms of the reaction is presented in Figure 33.

[0142] The ratio of products 1 to 2 can be adjusted by varying the concentrations of the templates. The forward:reverse template ratio may vary from, for example, molar ratios of 100:1, 75:1; 50:1, 40:1, 30:1, 20:1, 10:1, 5:1, 2.5:1, 1:1, 1:2.5, 1:5, 1:10, 1:20, 1:30, 1:40, 1:50, 1:75, or 1:100. The ratio of products (A to B) is dependent on the ratio of nicking enzyme to polymerase, i.e. a higher concentration of polymerase results in more of the longer length product (B) since there is comparatively less nicking enzyme to nick both strands simultaneously before the polymerase extends. Since displaced/released product of the reverse template feeds into the forward template and vice versa, exponential amplification is achieved. The nicking enzyme:polymerase ratio may vary from, for example, enzyme unit ratios of 20:1, 15:1; 10:1, 5:1, 4:1, 3:1, 2:1, 1.5:1, 1:1, 1:1.5, 1:2, 1:3, 1:4, 1:5, 1:10, 1:15, 1:20. In certain embodiments, the ratio of nicking enzyme to polymerase may, for example, be 1:3, 1:2, 1:1.5, or 1:0.8. Those of ordinary skill in the art recognize that these ratios may represent rounded values. This nicking and polymerase extension process continues until one of the resources (usually dNTPs or enzyme) is exhausted.

[0143] As demonstrated in the Examples, the time that the reaction is run may vary from, for example, within about 1 minute, or within about 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19 or 20 minutes. Longer reaction times may produce acceptable results where speed is not an issue. In some embodiments, the reaction is between 1-20 minutes, 1-15 minutes or 1-10, 1-8, 1-5, 1-2.5, 2.5-5, 2.5-8, 2.5-10, or 2.5-20 minutes in certain embodiments. The amplification processes described herein are efficient, and in some embodiments, as shown, for example, in the Examples, there is about $1x10^6$-fold or more amplification, about $1x10^7$-fold or more amplification, about $1x10^8$-fold or more amplification, about $1x10^9$-fold or more amplification, or about $1x10^{10}$-fold or more amplification in the time frame of the reaction, for example, in 5 or ten minutes. The reaction is highly sensitive, and is able to detect, for example, as low as about 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 20, 30, 40, 50, 60, 70, 80, 90, or 100 copies, or more, in a sample, as many as 200, 500, 1,000, 5,000, or 10,000, or more copies in a sample, or, for example, may detect a target that is present at a concentration of, for example, about 3.32E-13 micromolar to about 3.32E-8 micromolar, about 1.66E-12 micromolar to

about 3.32E-8 micromolar, about 3.32E-13 micromolar to about 3.32E-7 micromolar, or about 3.32E-13 micromolar to about 3.32E-6 micromolar.

[0144] In certain exemplary embodiments, methods are provided for amplifying a double stranded nucleic acid target sequence comprising contacting a target DNA molecule comprising a double-stranded target sequence, having a sense strand and an antisense strand, with a forward template and a reverse template, wherein the forward template comprises a nucleic acid sequence comprising a recognition region at the 3' end that is complementary or substantially complementary to the 3' end of the target sequence antisense strand; a nicking enzyme binding site and a nicking site upstream of the recognition region and a stabilizing region upstream of the nicking enzyme binding site and the nicking site; the reverse template comprises a nucleotide sequence comprising a recognition region at the 3' end that is complementary or substantially complementary to the 3' end of the target sequence sense strand, a nicking enzyme binding site and a nicking site upstream of the recognition region, and a stabilizing region upstream of the nicking enzyme binding site and the nicking site; providing a first nicking enzyme that is capable of nicking at the nicking site of the forward template, and does not nick within the target sequence; providing a second nicking enzyme that is capable of nicking at the nicking site of the reverse template and does not nick within the target sequence; and providing a DNA polymerase; under conditions wherein amplification is performed by multiple cycles of the polymerase extending the forward and reverse templates along the target sequence producing a double-stranded nicking site, and the nicking enzymes nicking at the nicking sites, producing an amplification product, wherein about 10^6 (1E +06) copies of a target sequence are produced in 10 minutes, under isothermal conditions. In other embodiments, about 10^7 (1E +07) copies are produced in 10 minutes. For multiplexed assays, the time to produce the same amount of copies may be increased to about, for example, 12, 14, 15, 18, or 20 minutes. The size of the target sequence in these assays, for purposes of calculating the efficiency, may be, for example, from about 20 to about 40 nucleotides, from 20 to 30 nucleotides, or, for example, from about 20 to about 33 nucleotides. The time of the reaction is calculated from the time that all of the reaction products are present in the same vessel, container, or the like, so that the amplification reaction may start, to the time that heat is applied or chemical agents are added to stop the reaction.

[0145] In another exemplary embodiment, methods are provided for amplifying a single-stranded nucleic acid target sequence, comprising contacting a target nucleic acid comprising a single-stranded target sequence with a reverse template, wherein the reverse template comprises a nucleotide sequence comprising a recognition region at the 3' end that is complementary or substantially complementary to the 3' end of the target sequence, a nicking enzyme binding site and a nicking site upstream of the recognition region, and a stabilizing region upstream of the nicking enzyme binding site and the nicking site; providing a first nicking enzyme that is capable of nicking at the nicking site of the reverse template, and does not nick within the target sequence; providing a DNA polymerase under conditions wherein the polymerase extends the reverse template along the target sequence; contacting the extended reverse template with a forward template, wherein the forward template comprises a recognition region at the 3'end that is identical to the 5'end of the target sequence a nicking enzyme binding site and a nicking site upstream of the recognition region, and a stabilizing region upstream of the nicking enzyme binding site and the nicking site; providing a second nicking enzyme that is capable of nicking at the nicking site of the forward template and does not nick within the target sequence; under conditions wherein amplification is performed by multiple cycles of the polymerase extending the forward and reverse templates along the target sequence producing a double-stranded nicking site, and the nicking enzymes nicking at the nicking sites, producing an amplification product, wherein about 10^6 (1E +06) copies of a target sequence are produced in 10 minutes, under isothermal conditions. In other embodiments, about 10^7 (1E +07) copies are produced in 10 minutes. For multiplexed assays, the time to produce the same amount of copies may be increased to about, for example, 12, 14, 15, 18, or 20 minutes. The size of the target sequence in these assays, for purposes of calculating the efficiency, may be, for example, from about 20 to about 40 nucleotides, or, for example, from about 20 to about 33 nucleotides. The time of the reaction is calculated from the time that all of the reaction products are present in the same vessel, container, or the like, so that the amplification reaction may start.

[0146] The present methods do not require the use of temperature cycling, as often is required in methods of amplification to dissociate the target sequence from the amplified nucleic acid. The temperature of the reaction may vary based on the length of the sequence, and the GC concentration, but, as understood by those of ordinary skill in the art, the temperature should be high enough to minimize non-specific binding. The temperature should also be suitable for the enzymes of the reaction, the nicking enzyme and the polymerase. For example, the reaction may be run at about 52°C, 53°C, 54°C, 55°C, 56°C, 57°C, 58°C, 59°C, or 60°C. In some embodiments, the reaction is run at about 37°C-85°C, 37°C -60°C, 54°C-60°C, 55°C-60°C, 58°C-60°C and, in exemplary embodiments, from 56°C-58°C. In certain embodiments, there is no denaturation step in the process. The entire amplification process, including interacting templates with target nucleic acid, is conducted within substantially isothermal conditions, and without a denaturing step (e.g., no significant temperature increase (e.g., no increase in temperature to 90-110 °C)), in some embodiments of the present methods.

[0147] Thus, in certain exemplary embodiments, methods are provided for amplifying a double stranded nucleic acid target sequence comprising contacting a target DNA molecule comprising a double- stranded target sequence, having

a sense strand and an antisense strand, with a forward template and a reverse template, wherein the forward template comprises a nucleic acid sequence comprising a recognition region at the 3' end that is complementary or substantially complementary to the 3' end of the target sequence antisense strand; a nicking enzyme binding site and a nicking site upstream of the recognition region and a stabilizing region upstream of the nicking enzyme binding site and the nicking site; the reverse template comprises a nucleotide sequence comprising a recognition region at the 3' end that is complementary or substantially complementary to the 3' end of the target sequence sense strand, a nicking enzyme binding site and a nicking site upstream of the recognition region, and a stabilizing region upstream of the nicking enzyme binding site and the nicking site; providing a first nicking enzyme that is capable of nicking at the nicking site of the forward template, and does not nick within the target sequence; providing a second nicking enzyme that is capable of nicking at the nicking site of the reverse template and does not nick within the target sequence; and providing a DNA polymerase; under conditions wherein amplification is performed by multiple cycles of the polymerase extending the forward and reverse templates along the target sequence producing a double- stranded nicking site, and the nicking enzymes nicking at the nicking sites, producing an amplification product, wherein the foregoing steps are conducted under isothermal conditions.

[0148] In another exemplary embodiment, methods are provided for amplifying a single-stranded nucleic acid target sequence, comprising contacting a target nucleic acid comprising a single-stranded target sequence with a reverse template, wherein the reverse template comprises a nucleotide sequence comprising a recognition region at the 3' end that is complementary or substantially complementary to the 3' end of the target sequence, a nicking enzyme binding site and a nicking site upstream of the recognition region, and a stabilizing region upstream of the nicking enzyme binding site and the nicking site; providing a first nicking enzyme that is capable of nicking at the nicking site of the reverse template, and does not nick within the target sequence; providing a DNA polymerase under conditions wherein the polymerase extends the reverse template along the target sequence; contacting the extended reverse template with a forward template, wherein the forward template comprises a recognition region at the 3'end that is identical to the 5'end of the target sequence a nicking enzyme binding site and a nicking site upstream of the recognition region, and a stabilizing region upstream of the nicking enzyme binding site and the nicking site; providing a second nicking enzyme that is capable of nicking at the nicking site of the forward template and does not nick within the target sequence; under conditions wherein amplification is performed by multiple cycles of the polymerase extending the forward and reverse templates along the target sequence producing a double-stranded nicking site, and the nicking enzymes nicking at the nicking sites, producing an amplification product, wherein the foregoing steps are conducted under isothermal conditions.

[0149] The polymerase may be mixed with the target nucleic acid molecule before, after, or at the same time as, the nicking enzyme. In exemplary embodiments, a reaction buffer is optimized to be suitable for both the nicking enzyme and the polymerase.

[0150] Reactions may be allowed to completion, that is, when one of the resources is exhausted. Or, the reaction may be stopped using methods known to those of ordinary skill in the art, such as, for example, heat inactivation, or the addition of EDTA, high salts, or detergents. In exemplary embodiments, where mass spectrometry is to be used following amplification, EDTA may be used to stop the reaction.

Reaction Components

[0151] In a 1.5 mL Eppendorf tube combine the following reagents in order from top to bottom:

Reagent Added:	microliters Per Reaction
H_2O	31.4
10X Thermopol Buffer (NEB)	5
10X NEB Buffer 3	2.5
100 mM $MgSO_4$	4.5
10 mM dNTPs	1.5
8 U/microliters Bst Pol	0.6
10 U/microliters N.BstNBI	1.5
20 micromolar Forward Template	0.25
20 micromolar Reverse Template	0.25

(continued)

Reagent Added:	microliters Per Reaction
Total reaction mixture	47.5
Target sample	2.5
Total Reaction Volume	50 microliters

[0152] The concentrations of components for the reaction conditions in this example are as follows:

Concentration	Component
45.7mM	Tris-HCl
13.9 mM	KCl
10 mM	$(NH_4)_2SO_4$
50 mM	NaCl
0.5 mM	DTT
15 mM	$MgCl_2$
0.10%	Triton X-100
0.008 mM	EDTA
6 μg/mL	BSA
3.90%	Glycerol (can be lower if using a more concentrated enzyme stock)
0.3 U/microliter	Nt.BstNBI
0.1-0.4 U/microliter	Bst polymerase (large fragment)
0.1 micromolar	Forward template
0.1 micromolar	Reverse template

[0153] Variations in buffer conditions, $MgSO_4$ concentration, polymerase concentration, and template concentrations all can be optimized based on the assay sequence and desired detection method. The amount of glycerol may, for example, be lowered if a more concentrated enzyme stock is used. In certain embodiments, the concentration of Mg^{2+} ions added as a reactant is about 9mM to about 25 mM, about 9mM to 21 mM, about 9 to 21 mM, about 9 to 20 mM, about 9 to 15 mM, and, in exemplary embodiments, about 10mM to about 18 mM, about 10mM to about 25 mM, about 10mM to 21 mM, about 12 to 21 mM, about 10 to 20 mM, about 10 to 15 mM, about 10.3 mM to about 20 mM, about 10.3 mM to about 14.9 mM, or about 15 mM, for example. Also, those of ordinary skill in the art recognize that the reaction may be run without EDTA or BSA; these components may be present in the reaction as part of the storage buffers for the enzymes. The volumes can be scaled for larger or smaller total reaction volumes. The volume is usually between 5 μL and 100 μL.

[0154] The template concentrations are typically in excess of the concentration of target. The concentrations of the forward and reverse templates can be at the same or at different concentrations to bias the amplification of one product over the other. The concentration of each is usually between 10 nM and 1μM.

[0155] Additives such as BSA, non-ionic detergents such as Triton X-100 or Tween-20, DMSO, DTT, and RNase inhibitor may be included for optimization purposes without adversely affecting the amplification reaction.

Preparing/Adding Target

[0156] Targets may be diluted in 1 x Thermopol Buffer II, 1 x TE (pH 7.5) or H_2O. Hot start conditions allow for faster, more specific amplification. In this case, the reaction mix (minus either enzymes or templates and target) is heated to the reaction temperature for 2 minutes, after which the reaction mix is added to the other component (enzymes or templates/target). The target can be added in any volume up to the total amount of water required in the reaction. In this case, the target would be diluted in water. In the example above for a 50 microliter total reaction volume, 2.5 microliters of the prepared target should be added per reaction to bring the total reaction volume to 50 microliters. Reaction volumes of the present methods can be increased or decreased, depending on the needs of the user. Reaction volumes of, for example, 5, 10, 15, 20, 25, 30, 35, 40, 45, 50 microliters or more, or larger reaction volumes of, for example, 75, 100, 150, 200, 300, 400, 500 microliters, for example, may be used in the present methods.

Running the Reaction

[0157] The reaction is run at a constant temperature, usually between 54°C and 60°C for the enzyme combination of Bst polymerase (large fragment) and Nt.Bst.NB1 nicking enzyme. Other enzyme combinations may be used and the optimal reaction temperature will be based on the optimal temperature for both the nicking enzyme and polymerase to work in concert as well as the melting temperature of the reaction products. The reaction is held at temperature for 2.5 to 10 minutes, for example, until the desired amount of amplification is achieved. The reaction may be stopped by either a heat inactivation step to inactivate the enzymes (when using enzymes that can be heat-killed). Alternatively, the reaction may be stopped by adding EDTA to the reaction.

Readout

[0158] The amplified target sequence may be detected by any method known to one of ordinary skill in the art. By way of non-limiting example, several of these known methods are presented herein. In one method, amplified products may be detected by gel electrophoresis, thus detecting reaction products having a specific length. The nucleotides may, for example, be labeled, such as, for example, with biotin. Biotin-labeled amplified sequences may be captured using avidin bound to a signal generating enzyme, for example, peroxidase.

[0159] Nucleic acid detection methods may employ the use of dyes that specifically stain double-stranded DNA. Intercalating dyes that exhibit enhanced fluorescence upon binding to DNA or RNA are a basic tool in molecular and cell biology. Dyes may be, for example, DNA or RNA intercalating fluorophores and may include but are not limited to the following examples: Acridine orange, ethidium bromide, Hoechst dyes, PicoGreen, propidium iodide, SYBR I (an asymmetrical cyanine dye), SYBR II, TOTO (a thiaxole orange dimer) and YOYO (an oxazole yellow dimer), and the like. Dyes provide an opportunity for increasing the sensitivity of nucleic acid detection when used in conjunction with various detection methods and may have varying optimal usage parameters. For example ethidium bromide is commonly used to stain DNA in agarose gels after gel electrophoresis and during PCR (Hiquchi et al., Nature Biotechnology 10; 413-417, April 1992), propidium iodide and Hoechst 33258 are used in flow cytometry to determine DNA ploidy of cells, SYBR Green 1 has been used in the analysis of double-stranded DNA by capillary electrophoresis with laser induced fluorescence detection and Pico Green has been used to enhance the detection of double-stranded DNA after matched ion pair polynucleotide chromatography (Singer et al., Analytical Biochemistry 249, 229-238 1997).

[0160] Nucleic acid detection methods may also employ the use of labeled nucleotides incorporated directly into the target sequence or into probes containing complementary or substantially complementary sequences to the target of interest. Such labels may be radioactive and/or fluorescent in nature and can be resolved in any of the manners discussed herein. Labeled nucleotides, which can be detected but otherwise function as native nucleotides, are to be distinguished from modified nucleotides, which do not function as native nucleotides.

[0161] Methods of detecting and/or continuously monitoring the amplification of nucleic acid products are also well known to those skilled in the art and several examples are described below.

[0162] The production or presence of target nucleic acids and nucleic acid sequences may be detected and monitored by Molecular Beacons. Molecular Beacons are hair-pin shaped oligonucleotides containing a fluorophore on one end and a quenching dye on the opposite end. The loop of the hair-pin contains a probe sequence that is complementary or substantially complementary to a target sequence and the stem is formed by annealing of complementary or substantially complementary arm sequences located on either side of the probe sequence. A fluorophore and a quenching molecule are covalently linked at opposite ends of each arm. Under conditions that prevent the oligonucleotides from hybridizing to its complementary or substantially complementary target or when the molecular beacon is free in solution the fluorescent and quenching molecules are proximal to one another preventing fluorescence resonance energy transfer (FRET). When the molecular beacon encounters a target molecule, hybridization occurs; the loop structure is converted to a stable more rigid conformation causing separation of the fluorophore and quencher molecules leading to fluorescence (Tyagi et al. Nature Biotechnology 14: March 1996, 303-308). Due to the specificity of the probe, the generation of fluorescence is exclusively due to the synthesis of the intended amplified product.

[0163] Molecular beacons are extraordinarily specific and can discern a single nucleotide polymorphism. Molecular beacons can also be synthesized with different colored fluorophores and different target sequences, enabling several products in the same reaction to be quantified simultaneously. For quantitative amplification processes, molecular beacons can specifically bind to the amplified target following each cycle of amplification, and because non-hybridized molecular beacons are dark, it is not necessary to isolate the probe-target hybrids to quantitatively determine the amount of amplified product. The resulting signal is proportional to the amount of amplified product. This can be done in real time. As with other real time formats, the specific reaction conditions must be optimized for each primer/probe set to ensure accuracy and precision.

[0164] The production or presence of target nucleic acids and nucleic acid sequences may also be detected and monitored by Fluorescence resonance energy transfer (FRET). FRET is an energy transfer mechanism between two

chromophores: a donor and an acceptor molecule. Briefly, a donor fluorophore molecule is excited at a specific excitation wavelength. The subsequent emission from the donor molecule as it returns to its ground state may transfer excitation energy to the acceptor molecule through a long range dipoledipole interaction. The intensity of the emission of the acceptor molecule can be monitored and is a function of the distance between the donor and the acceptor, the overlap of the donor emission spectrum and the acceptor absorption spectrum and the orientation of the donor emission dipole moment and the acceptor absorption dipole moment. FRET is a useful tool to quantify molecular dynamics, for example, in DNA-DNA interactions as seen with Molecular Beacons. For monitoring the production of a specific product a probe can be labeled with a donor molecule on one end and an acceptor molecule on the other. Probe-target hybridization brings a change in the distance or orientation of the donor and acceptor and FRET change is observed. (Joseph R. Lakowicz, "Principles of Fluorescence Spectroscopy", Plenum Publishing Corporation, 2nd edition (July 1, 1999)).

[0165] The production or presence of target nucleic acids and nucleic acid sequences may also be detected and monitored by Mass Spectrometry. Mass Spectrometry is an analytical technique that may be used to determine the structure and quantity of the target nucleic acid species and can be used to provide rapid analysis of complex mixtures. Following the method, samples are ionized, the resulting ions separated in electric and/or magnetic fields according to their mass- to- charge ratio, and a detector measures the mass- to- charge ratio of ions. (Crain, P. F. and McCloskey, J. A., Current Opinion in Biotechnology 9: 25- 34 (1998)) . Mass spectrometry methods include, for example, MALDI, MALDI/TOF, or Electrospray. These methods may be combined with gas chromatography (GC/MS) and liquid chromatography (LC/MS) . MS has been applied to the sequence determination of DNA and RNA oligonucleotides (Limbach P., MassSpectrom. Rev. 15: 297- 336 (1996) ; Murray K., J. Mass Spectrom. 31: 1203- 1215 (1996)) . MS and more particularly, matrix- assisted laser desorption/ ionization MS (MALDI MS) has the potential of very high throughput due to high- speed signal acquisition and automated analysis off solid surfaces. It has been pointed out that MS, in addition to saving time, measures an intrinsic property of the molecules, and therefore yields a significantly more informative signal (Koster H. et al., Nature Biotechnol., 14: 1123- 1128 (1996)) .

The production or presence of target nucleic acids and nucleic acid sequences may also be detected and monitored by various methods of gel electrophoresis. Gel electrophoresis involves the separation of nucleic acids through a matrix, generally a cross- linked polymer, using an electromotive force that pulls the molecules through the matrix. Molecules move through the matrix at different rates causing a separation between products that can be visualized and interpreted via any one of a number of methods including but not limited to; autoradiography, phosphorimaging, and staining with nucleic acid chelating dyes.

The production or presence of target nucleic acids and nucleic acid sequences may also be detected and monitored by capillary gel electrophoresis. Capillary- gel Electrophoresis (CGE) is a combination of traditional gel electrophoresis and liquid chromatography that employs a medium such as polyacrylamide in a narrow bore capillary to generate fast, high-efficient separations of nucleic acid molecules with up to single base resolution. CGE is commonly combined with laser induced fluorescence (LIF) detection where as few as six molecules of stained DNA can be detected. CGE/LIF detection generally involves the use of fluorescent DNA intercalating dyes including ethidium bromide, YOYO and SYBR Green 1 but can also involve the use of fluorescent DNA derivatives where the fluorescent dye is covalently bound to the DNA. Simultaneous identification of several different target sequences can be made using this method.

The production or presence of target nucleic acids and nucleic acid sequences may also be detected and monitored by various surface capture methods. This is accomplished by the immobilization of specific oligonucleotides to a surface producing a biosensor that is both highly sensitive and selective. Surfaces used in this method may include but are not limited to gold and carbon and may use a number of covalent or noncovalent coupling methods to attach the probe to the surface. The subsequent detection of a target DNA can be monitored by a variety of methods.

Electrochemical methods generally involve measuring the cathodic peak of intercalators, such as methylene blue, on the DNA probe electrode and visualized with square wave voltammograms. Binding of the target sequence can be observed by a decrease in the magnitude of the voltammetric reduction signals of methylene blue as it interacts with dsDNA and ssDNA differently reflecting the extent of the hybrid formation.

Surface Plasmon Resonance (SPR) can also be used to monitor the kinetics of probe attachment as well as the process of target capture. SPR does not require the use of fluorescence probes or other labels. SPR relies on the principle of light being reflected and refracted on an interface of two transparent media of different refractive indexes. Using monochromatic and p- polarized light and two transparent media with an interface comprising a thin layer of gold, total reflection of light is observed beyond a critical angle, however the electromagnetic field component of the light penetrates into the medium of lower refractive index creating an evanescent wave and a sharp shadow (surface plasmon resonance) . This is due to the resonance energy transfer between the wave and the surface plasmons. The resonance conditions are influenced by the material absorbed on the thin metal film and nucleic acid molecules, proteins and sugars concentrations are able to be measured based on the relation between resonance units and mass concentration.

The production or presence of target nucleic acids and nucleic acid sequences may also be detected and monitored by lateral flow devices. Lateral Flow devices are well known. These devices generally include a solid phase fluid permeable flow path through which fluid flows through by capillary force. Examples include, but are not limited to, dipstick assays

and thin layer chromatographic plates with various appropriate coatings. Immobilized on the flow path are various binding reagents for the sample, binding partners or conjugates involving binding partners for the sample and signal producing systems. Detection of samples can be achieved in several manners; enzymatic detection, nanoparticle detection, colorimetric detection, and fluorescence detection, for example. Enzymatic detection may involve enzyme-labeled probes that are hybridized to complementary or substantially complementary nucleic aid targets on the surface of the lateral flow device. The resulting complex can be treated with appropriate markers to develop a readable signal. Nanoparticle detection involves bead technology that may use colloidal gold, latex and paramagnetic nanoparticles. In one example, beads may be conjugated to an anti- biotin antibody. Target sequences may be directly biotinylated, or target sequences may be hybridized to a sequence specific biotinylated probes. Gold and latex give rise to colorimetric signals visible to the naked eye and paramagnetic particles give rise to a non- visual signal when excited in a magnetic field and can be interpreted by a specialized reader.

[0166] Fluorescence- based lateral flow detection methods are also known, for example, dual fluorescein and biotin-labeled oligo probe methods, UPT- NALF utilizing up- converting phosphor reporters composed of lanthanide elements embedded in a crystal (Corstjens et al., Clinical Chemistry, 47: 10, 1885- 1893, 2001), as well as the use of quantum dots.

[0167] Nucleic acids can also be captured on lateral flow devices. Means of capture may include antibody dependent and antibody independent methods. Antibody-dependent capture generally comprises an antibody capture line and a labeled probe that is complementary or substantially complementary sequence to the target. Antibody-independent capture generally uses non-covalent interactions between two binding partners, for example, the high affinity and irreversible linkage between a biotinylated probe and a streptavidin line. Capture probes may be immobilized directly on lateral flow membranes. Both antibody dependent and antibody independent methods may be used in multiplexing.

[0168] The production or presence of target nucleic acids and nucleic acid sequences may also be detected and monitored by multiplex DNA sequencing. Multiplex DNA sequencing is a means of identifying target DNA sequences from a pool of DNA. The technique allows for the simultaneous processing of many sequencing templates. Pooled multiple templates can be resolved into individual sequences at the completion of processing. Briefly, DNA molecules are pooled, amplified and chemically fragmented. Products are fractionated by size on sequencing gels and transferred to nylon membranes. The membranes are probed and autoradiographed using methods similar to those used in standard DNA sequencing techniques (Church et al., Science 1998 Apr 8; 240 (4849) : 185- 188) . Autoradiographs can be evaluated and the presence of target nucleic acid sequence can be quantified.

Kits

[0169] Kits used for the present methods may comprise, for example, one or more polymerases, forward and reverse templates, and one or more nicking enzymes, as described herein. Where one target is to be amplified, one or two nicking enzymes may be included in the kit. Where multiple target sequences are to be amplified, and the templates designed for those target sequences comprise the nicking enzyme binding sites for the same nicking enzyme, then one or two nicking enzymes may be included. Or, where the templates are recognized by different nicking enzymes, more nicking enzymes may be included in the kit, such as, for example, 3 or more.

[0170] The kits used for the present methods may also comprise one or more of the components in any number of separate containers, packets, tubes, vials, microtiter plates and the like, or the components may be combined in various combinations in such containers.

[0171] The components of the kit may, for example, be present in one or more containers, for example, all of the components may be in one container, or, for example, the enzymes may be in a separate container from the templates. The components may, for example, be lyophilized, freeze dried, or in a stable buffer. In one example, the polymerase and nicking enzymes are in lyophilized form in a single container, and the templates are either lyophilized, freeze dried, or in buffer, in a different container. Or, in another example, the polymerase, nicking enzymes, and the templates are, in lyophilized form, in a single container. Or, the polymerase and the nicking enzyme may be separated into different containers.

[0172] Kits may further comprise, for example, dNTPs used in the reaction, or modified nucleotides, cuvettes or other containers used for the reaction, or a vial of water or buffer for re-hydrating lyophilized components. The buffer used may, for example, be appropriate for both polymerase and nicking enzyme activity.

[0173] The kits used for the present methods may also comprise instructions for performing one or more methods described herein and/or a description of one or more compositions or reagents described herein. Instructions and/or descriptions may be in printed form and may be included in a kit insert. A kit also may include a written description of an Internet location that provides such instructions or descriptions.

[0174] Kits may further comprise reagents used for detection methods, such as, for example, reagents used for FRET, lateral flow devices, dipsticks, fluorescent dye, colloidal gold particles, latex particles, a molecular beacon, or polystyrene beads.

[0175] An advantage of the present methods and the present kits is that they can be used in any device that provides

a constant temperature, including thermocyclers, incubation ovens, water baths, and heat blocks.

[0176] Thus, provided in the present methods is method for nucleotide sequence amplification, which comprises: combining a target nucleic acid having a target nucleotide sequence with (i) a polymerase, (ii) a first template nucleic acid that hybridizes to the a first strand of the target nucleotide sequence, and (iii) a second template nucleic acid that hybridizes to the complement of the first strand of the target nucleotide sequence, in an amplification reaction, under conditions in which the polymerase extends the template nucleic acids, thereby generating extended template nucleic acid amplicons; wherein: the target nucleotide sequence is between 20 and 40 nucleotides in length; the target nucleotide sequence is amplified 1E+6- fold or more in about ten minutes; and the foregoing steps are conducted under substantially isothermal conditions.

[0177] Also provided is a method for nucleotide sequence amplification, which comprises: combining a target nucleic acid having a target nucleotide sequence with (i) a polymerase, (ii) a first template nucleic acid that hybridizes to the a first strand of the target nucleotide sequence, and (iii) a second template nucleic acid that hybridizes to the complement of the first strand of the target nucleotide sequence, in an amplification reaction, under conditions in which the polymerase extends the template nucleic acids, thereby generating extended template nucleic acid amplicons; wherein:the target nucleotide sequence is between 20 and 40 nucleotides in length; the first template comprises a nucleic acid sequence comprising a first template recognition region at the 3' end that is complementary or substantially complementary to the 3' end of the first strand of the target nucleotide sequence; the second template comprises a nucleotide sequence comprising a second template recognition region at the 3' end that is complementary or substantially complementary to the 3' end of the complement of the first strand of the target nucleotide sequence; the target nucleotide sequence comprises from 1 to 5 nucleotides more than the sum of the nucleotides of the first template recognition region and the second template recognition region; the target nucleotide sequence is amplified 1E+6-fold or more in about ten minutes; and the foregoing steps are conducted under substantially isothermal conditions.

[0178] Also provided is a method for nucleotide sequence amplification, which comprises: combining a target nucleic acid having a target nucleotide sequence with (i) a polymerase, (ii) a first template nucleic acid that hybridizes to the a first strand of the target nucleotide sequence, and (iii) a second template nucleic acid that hybridizes to the complement of the first strand of the target nucleotide sequence, in an amplification reaction, under conditions in which the polymerase extends the template nucleic acids, thereby generating extended template nucleic acid amplicons; wherein:the first template comprises a nucleic acid sequence comprising a first template recognition region at the 3' end that is complementary or substantially complementary to the 3' end of the first strand of the target nucleotide sequence, wherein the recognition region is 8-15 nucleotides long; the second template comprises a nucleotide sequence comprising a second template recognition region at the 3' end that is complementary or substantially complementary to the 3' end of the complement of the first strand of the target nucleotide sequence, wherein the recognition region is 8-15 nucleotides long; the target nucleotide sequence is amplified 1E+6-fold or more in about ten minutes; and the foregoing steps are conducted under substantially isothermal conditions.

[0179] In certain aspects of the present methods, the first template comprises a nucleic acid sequence comprising a first template recognition region at the 3' end that is complementary or substantially complementary to the 3' end of the first strand of the target nucleotide sequence; and the second template comprises a nucleotide sequence comprising a second template recognition region at the 3' end that is complementary or substantially complementary to the 3' end of the complement of the first strand of the target nucleotide sequence. In certain aspects, the target nucleotide sequence comprises from 1 to 5 nucleotides more than the sum of the nucleotides of the first template recognition region and the second template recognition region. In other aspects, the first template and second templates comprise nicking enzyme binding sites and nicking sites upstream of the recognition regions, and the amplification reaction further comprises one or more nicking enzymes that are capable of nicking at the nicking site of said forward and said reverse templates, wherein either one nicking enzyme is capable of nicking both of said templates, or each template is capable of being nicked by at least one of the nicking enzymes, and wherein said one or more nicking enzymes do not nick within said target sequence.

[0180] In some embodiments, the target nucleotide sequence comprises 1 nucleotide more than the sum of the nucleotides of the first template recognition region and the second template recognition region. In other embodiments, the target nucleotide sequence comprises 2 nucleotides more than the sum of the nucleotides of the first template recognition region and the second template recognition region. In yet other embodiments, the target nucleotide sequence comprises 3 nucleotides more than the sum of the nucleotides of the first template recognition region and the second template recognition region.

[0181] In certain aspects of the present methods, the target nucleic acid is double stranded or single stranded. In certain aspects, the target nucleic acid is double-stranded DNA. In other aspects, the target nucleic acid is single-stranded DNA. In yet other aspects, the target nucleic acid is RNA. The target nucleic acid may be, for example, selected from the group consisting of genomic DNA, plasmid DNA, viral DNA, mitochondrial DNA, and synthetic double-stranded DNA. The target nucleic acid may be, for example, selected from the group consisting of viral DNA, cDNA, and synthetic single-stranded DNA. The target nucleic acid may be, for example, selected from the group consisting of messenger RNA,

viral RNA, ribosomal RNA, transfer RNA, micro RNA, micro RNA precursor, and synthetic RNA.

[0182] In the present methods, the DNA polymerase may be, for example, a thermophilic polymerase. The polymerase may, for example, be selected from the group consisting of Bst (large fragment), 9°N, $Vent_R^{®}$ (exo-) DNA Polymerase, Therminator, and Therminator II. In certain aspects, the polymerase is Bst (large fragment).

[0183] In certain embodiments, the first and second templates comprise nicking enzyme binding sites recognized by the same nicking enzyme and said first and said second nicking enzyme are the same. The nicking enzymes may be, for example, selected from the group consisting of Nt.BspQI, Nb.BbvCi, Nb.BsmI, Nb.BsrDI, Nb.BtsI, Nt.AlwI, Nt.BbvCI, Nt.BstNBI, Nt.CviPII, Nb.Bpu10I, and Nt.Bpu10I.

[0184] In some aspects of the present method, the portion of the nucleic acid sequence of the first strand that is complementary or substantially complementary to the first strand of the target nucleotide sequence is 8-15 nucleotides in length and wherein the portion of the second strand that is complementary or substantially complementary to the target nucleotide sequence is 8-15 nucleotides in length. In some aspects, the first template is provided at the same concentration as the second template. In other aspects, one of the first or second templates is provided at a ratio to the other template at the range of ratios of 1:100 to 100:1. The reactions of the present methods may further comprise a second polymerase. In some aspects, at least one of the first or second polymerases comprises reverse transcriptase activity.

[0185] In certain embodiments of the present method, the amplification is conducted between 54°C and 60°C. In other embodiments, the amplification is conducted between 56°C and 58°C. In certain embodiments, wherein the amplification reaction is held at a constant temperature for 1 to 10 minutes. In other embodiments, the amplification reaction is held at a constant temperature for 1 to 20 minutes.

[0186] The present method may further comprise detecting the amplification product. Thus, in certain aspects, the amplification product is detected by detection method selected from the group consisting of gel electrophoresis, mass spectrometry, SYBR I fluorescence, SYBR II fluorescence, SYBR Gold, Pico Green, TOTO-3, intercalating dye detection, fluorescence resonance energy transfer (FRET), molecular beacon detection, surface capture, capillary electrophoresis, incorporation of labeled nucleotides to allow detection by capture, fluorescence polarization, and lateral flow capture.

[0187] In some aspects, at least two target sequences are capable of being amplified. In certain aspects, the amplification products are detected on a solid surface. In some aspects, at least one capture probe is immobilized on a solid surface. In some embodiments, at least one of said templates comprises a spacer, blocking group, or a modified nucleotide.

[0188] In certain embodiments of the present methods, the target nucleotide sequence is amplified 1E+6-fold or more in about five minutes. In other embodiments, the target nucleotide sequence is amplified 1E+6-fold or more in about 2.5 minutes. In other embodiments, the target nucleotide sequence is amplified 1E+7-fold or more in about five minutes. In other embodiments, the target nucleotide sequence is amplified 1E+8-fold or more in about five minutes. In yet other embodiments, wherein the target nucleotide sequence is amplified 1E+9-fold or more in about five minutes.

[0189] The present methods also include a method for amplifying a double-stranded nucleic acid target sequence, comprising contacting a target DNA molecule comprising a double-stranded target sequence, having a sense strand and an antisense strand, with a forward template and a reverse template, wherein said forward template comprises a nucleic acid sequence comprising a recognition region at the 3' end that is complementary to the 3' end of the target sequence antisense strand; a nicking enzyme binding site and a nicking site upstream of said recognition region and a stabilizing region upstream of said nicking site, wherein the portion of the nucleic acid sequence that is complementary to the 3' end of the target antisense strand is 8-15 nucleotides in length; said reverse template comprises a nucleotide sequence comprising recognition region at the 3' end that is complementary to the 3' end of the target sequence sense strand, a nicking enzyme binding site and a nicking site upstream of said recognition region, and a stabilizing region upstream of said nicking site, wherein the portion of the nucleic acid sequence that is complementary to the 3' end of the target antisense strand is 8-15 nucleotides in length; providing a first nicking enzyme that is capable of nicking upstream, downstream, or at the nicking site of said forward template, and does not nick within said target sequence; providing a second nicking enzyme that is capable of nicking upstream, downstream, or at the nicking site of said reverse template and does not nick within said target sequence; and providing a DNA polymerase;

under essentially isothermal conditions, wherein amplification is performed by multiple cycles of said polymerase extending said forward and reverse templates along said target sequence producing a double-stranded nicking site, and said nicking enzymes nicking at said nicking sites, or amplified copies of said sites, producing an amplification product.

[0190] Also provided is a method for amplifying a single-stranded nucleic acid target sequence, comprising contacting a target nucleic acid comprising a single-stranded target sequence with a reverse template, wherein said reverse template comprises a nucleic acid sequence comprising a recognition region at the 3' end that is complementary to the 3' end of the target sequence, a nicking enzyme binding site and a nicking site upstream of said recognition region, and a stabilizing region upstream of said nicking site, wherein the portion of the nucleic acid sequence that is complementary to the 3' end of the target sequence is 8-15 nucleotides in length; providing a first nicking enzyme that is capable of nicking at the nicking site of said reverse template, and does not nick within said target sequence; providing a DNA polymerase

under conditions wherein said polymerase extends said reverse template along said target sequence; contacting said extended reverse template with a forward template, wherein said forward template comprises a recognition region at the 3'end that is complementary to the 3' end of the extended reverse template, a nicking enzyme binding site and a nicking site upstream of said recognition region, and a stabilizing region upstream of said nicking site, wherein the portion of the nucleic acid sequence that is complementary to the 3' end of the target antisense strand is 8-15 nucleotides in length; providing a second nicking enzyme that is capable of nicking at the nicking site of said forward template and does not nick within said target sequence or within the complement of said target sequence; wherein the amplification is conducted under essentially isothermal conditions wherein amplification is performed by multiple cycles of said polymerase extending said forward and reverse templates along said target sequence, producing double-stranded nicking sites, and said nicking enzymes nicking at said nicking sites, producing an amplification product. In some aspects of the present method, the DNA polymerase is a thermophilic polymerase. For example, the polymerase may be selected from the group consisting of Bst (large fragment), 9°N, $Vent_R$® (exo-) DNA Polymerase, Therminator, and Therminator II. In certain aspects, the polymerase is Bst(large fragment).

[0191] In certain aspects, the nicking enzymes nick downstream of the nicking enzyme binding site. In other aspects, the forward and reverse templates comprise nicking enzyme binding sites recognized by the same nicking enzyme and said first and said second nicking enzymes are the same. In certain aspects, the nicking enzymes are selected from the group consisting of Nt.BspQI, Nb.BbvCi, Nb.BsmI, Nb.BsrDI, Nb.BtsI, Nt.AlwI, Nt.BbvCI, Nt.BstNBI, Nt.CviPII, Nb.Bpu10I, and Nt.Bpu10I.

[0192] In some embodiments of the present methods, the target sequence comprises from 1 to 5 nucleotides more than the sum of the nucleotides of said forward template recognition region and said reverse template recognition region. In certain embodiments, the target sequence comprises 1 nucleotide more than the sum of the nucleotides of said forward template recognition region and said reverse template recognition region. In other embodiments, the target sequence comprises 2 nucleotides more than the sum of the nucleotides of said forward template recognition region and said reverse template recognition region.

[0193] In certain aspects, the target DNA molecule is selected from the group consisting of genomic DNA, plasmid, mitochondrial, and viral DNA. In other aspects, the target nucleic acid is selected from the group consisting of viral DNA, messenger RNA, microRNA, and microRNA precursors. In other aspects, the forward template is provided at the same concentration as the reverse template. In yet other aspects, one of the forward or reverse templates is provided at a ratio to the other template at the range of ratios of 1:100 to 100:1 1

[0194] In certain embodiments, the present method further comprises a second polymerase. For example, at least one of the polymerases may comprise reverse transcriptase activity. In certain aspects, the amplification is conducted between 54°C and 60°C. In other aspects, the amplification reaction is held at a constant temperature for 1 to 10 minutes.

[0195] The present method may further comprise detecting the amplification product. For example, the amplification product may be detected by a method selected from the group consisting of gel electrophoresis, mass spectrometry, SYBR I fluorescence, SYBR II fluorescence, SYBR Gold, Pico Green, TOTO-3, intercalating dye detection, FRET, molecular beacon detection, surface capture, capillary electrophoresis, incorporation of labeled nucleotides to allow detection by capture, fluorescence polarization, and lateral flow capture.

[0196] In certain aspects, at least two target sequences are capable of being amplified. In other aspects, the amplification products are detected on a solid surface. In some aspects, at least one capture probe is immobilized on a solid surface. In other aspects, at least one of said templates comprises a spacer, blocking group, or a modified nucleotide.

[0197] The present methods also include a method for amplifying a double-stranded nucleic acid target sequence, comprising contacting a target DNA molecule comprising a double-stranded target sequence, having a sense strand and an antisense strand, with a forward template and a reverse template, wherein said forward template comprises a nucleic acid sequence comprising a recognition region at the 3' end that is complementary to the 3' end of the target sequence antisense strand; a nicking enzyme binding site and a nicking site upstream of said recognition region and a stabilizing region upstream of said nicking site; said reverse template comprises a nucleotide sequence comprising a recognition region at the 3' end that is complementary to the 3' end of the target sequence sense strand, a nicking enzyme binding site and a nicking site upstream of said recognition region, and a stabilizing region upstream of said nicking site; and said target sequence comprises from 1 to 5 nucleotides more than the sum of the nucleotides of said forward template recognition region and said reverse template recognition region; providing a first nicking enzyme that is capable of nicking at the nicking site of said forward template, and does not nick within said target sequence; providing a second nicking enzyme that is capable of nicking at the nicking site of said reverse template and does not nick within said target sequence; and providing a DNA polymerase; under essentially isothermal conditions, wherein amplification is performed by multiple cycles of said polymerase extending said forward and reverse templates along said target sequence producing a double-stranded nicking site, and said nicking enzymes nicking at said nicking sites, or amplified copies of said sites, producing an amplification product.

[0198] Also provided is a method for amplifying a single-stranded nucleic acid target sequence, comprising contacting a target nucleic acid comprising a single-stranded target sequence with a reverse template, wherein said reverse template

comprises a nucleic acid sequence comprising a recognition region at the 3' end that is complementary to the 3' end of the target sequence, a nicking enzyme binding site and a nicking site upstream of said recognition region, and a stabilizing region upstream of said nicking site, wherein the portion of the nucleic acid sequence that is complementary to the 3' end of the target sequence is 8-15 nucleotides in length; providing a first nicking enzyme that is capable of nicking at the nicking site of said reverse template, and does not nick within said target sequence; providing a DNA polymerase under conditions wherein said polymerase extends said reverse template along said target sequence; contacting said extended reverse template with a forward template, wherein said forward template comprises a recognition region at the 3'end that is complementary to the 3' end of the extended reverse template, a nicking enzyme binding site and a nicking site upstream of said recognition region, and a stabilizing region upstream of said nicking site, wherein said target sequence comprises from 1 to 5 nucleotides more than the sum of the nucleotides of said forward template recognition region and said reverse template recognition region; providing a second nicking enzyme that is capable of nicking at the nicking site of said forward template and does not nick within said target sequence or within the complement of said target sequence; wherein the amplification is conducted under essentially isothermal conditions wherein amplification is performed by multiple cycles of said polymerase extending said forward and reverse templates along said target sequence, producing double-stranded nicking sites, and said nicking enzymes nicking at said nicking sites, producing an amplification product.

[0199] In certain embodiments, the target sequence comprises 1 nucleotide more than the sum of the nucleotides of said forward template recognition region and said reverse template recognition region. In other embodiments, the target sequence comprises 2 nucleotides more than the sum of the nucleotides of said forward template recognition region and said reverse template recognition region. In other embodiments, the target sequence comprises 3 nucleotides more than the sum of the nucleotides of said forward template recognition region and said reverse template recognition region.

[0200] Also provided is a method for amplifying a double-stranded nucleic acid target sequence, comprising contacting a target DNA molecule comprising a double-stranded target sequence, having a sense strand and an antisense strand, with a forward template and a reverse template, wherein said forward template comprises a nucleic acid sequence comprising a recognition region at the 3' end that is complementary to the 3' end of the target sequence antisense strand; a nicking enzyme binding site and a nicking site upstream of said recognition region and a stabilizing region upstream of said nicking site; said reverse template comprises a nucleotide sequence comprising a recognition region at the 3' end that is complementary to the 3' end of the target sequence sense strand, a nicking enzyme binding site and a nicking site upstream of said recognition region, and a stabilizing region upstream of said nicking site; providing a first nicking enzyme that is capable of nicking at the nicking site of said forward template, and does not nick within said target sequence; providing a second nicking enzyme that is capable of nicking at the nicking site of said reverse template and does not nick within said target sequence; and providing a DNA polymerase; under essentially isothermal conditions, wherein amplification is performed by multiple cycles of said polymerase extending said forward and reverse templates along said target sequence producing a double-stranded nicking site, and said nicking enzymes nicking at said nicking sites, or amplified copies of said sites, producing an amplification product.

[0201] Also provided is method for amplifying a double-stranded nucleic acid target sequence, comprising contacting a target DNA molecule comprising a double-stranded target sequence, having a sense strand and an antisense strand, with a forward template and a reverse template, wherein said forward template comprises a nucleic acid sequence comprising a recognition region at the 3' end that is complementary to the 3' end of the target sequence antisense strand; a nicking enzyme binding site and a nicking site upstream of said recognition region and a stabilizing region upstream of said nicking site; said reverse template comprises a nucleotide sequence comprising recognition region at the 3' end that is complementary to the 3' end of the target sequence sense strand, a nicking enzyme binding site and a nicking site upstream of said recognition region, and a stabilizing region upstream of said nicking site; providing a first nicking enzyme that is capable of nicking upstream, downstream, or at the nicking site of said forward template, and does not nick within said target sequence; providing a second nicking enzyme that is capable of nicking upstream, downstream, or at the nicking site of said reverse template and does not nick within said target sequence; and providing a DNA polymerase;under essentially isothermal conditions, wherein amplification is performed by multiple cycles of said polymerase extending said forward and reverse templates along said target sequence producing a double-stranded nicking site, and said nicking enzymes nicking at said nicking sites, or amplified copies of said sites, producing an amplification product.

[0202] Also provided is a method for amplifying a double-stranded nucleic acid target sequence, comprising contacting a target DNA molecule comprising a double-stranded target sequence, having a sense strand and an antisense strand, with a forward template and a reverse template, wherein said forward template comprises a nucleic acid sequence comprising a recognition region at the 3' end that is complementary to the 3' end of the target sequence antisense strand; a nicking enzyme binding site and a nicking site upstream of said recognition region and a stabilizing region upstream of said nicking site; said reverse template comprises a nucleotide sequence comprising a recognition region at the 3' end that is complementary to the 3' end of the target sequence sense strand, a nicking enzyme binding site and a nicking site upstream of said recognition region, and a stabilizing region upstream of said nicking site; providing a first nicking

enzyme that is capable of nicking at the nicking site of said forward template, and does not nick within said target sequence; providing a second nicking enzyme that is capable of nicking at the nicking site of said reverse template and does not nick within said target sequence; and providing a DNA polymerase; under essentially isothermal conditions, wherein amplification is performed by multiple cycles of said polymerase extending said forward and reverse templates along said target sequence producing a double-stranded nicking site, and said nicking enzymes nicking at said nicking sites, or amplified copies of said sites, producing an amplification product, wherein at least a 1E + 7 fold amplification of a 22-35 nucleotide long target sequence is obtained when the amplification reaction is run for twelve minutes.

[0203] The present method also provides a method for amplifying a double-stranded nucleic acid target sequence, comprising contacting a target DNA molecule comprising a double-stranded target sequence, having a sense strand and an antisense strand, with a forward template and a reverse template, wherein said forward template comprises a nucleic acid sequence comprising a recognition region at the 3' end that is complementary to the 3' end of the target sequence antisense strand; a nicking enzyme binding site and a nicking site upstream of said recognition region and a stabilizing region upstream of said nicking site, wherein the portion of the nucleic acid sequence that is complementary to the 3' end of the target antisense strand is 8-15 nucleotides in length; said reverse template comprises a nucleotide sequence comprising recognition region at the 3' end that is complementary to the 3' end of the target sequence sense strand, a nicking enzyme binding site and a nicking site upstream of said recognition region, and a stabilizing region upstream of said nicking site, wherein the portion of the nucleic acid sequence that is complementary to the 3' end of the target antisense strand is 8-15 nucleotides in length; providing a first nicking enzyme that is capable of nicking upstream, downstream, or at the nicking site of said forward template, and does not nick within said target sequence; providing a second nicking enzyme that is capable of nicking upstream, downstream, or at the nicking site of said reverse template and does not nick within said target sequence; and providing a DNA polymerase, under essentially isothermal conditions, wherein amplification is performed by multiple cycles of said polymerase extending said forward and reverse templates along said target sequence producing a double-stranded nicking site, and said nicking enzymes nicking at said nicking sites, or amplified copies of said sites, producing an amplification product, wherein at least a 1E + 7 fold amplification of a 22-35 nucleotide long target sequence is obtained when the amplification reaction is run for twelve minutes.

[0204] Also provided are kits for amplifying a nucleic acid target sequence, comprising a DNA polymerase; a first template for nucleic acid amplification, comprising a recognition region at the 3' end that is complementary to the 3' end of a target sequence sense strand; a nicking enzyme binding site and a nicking site upstream of said recognition region; and a stabilizing region upstream of said nicking site, wherein the portion of the nucleic acid sequence that is complementary to the 3' end of the target sequence sense strand is 8-15 nucleotides in length; a second template for nucleic acid amplification, comprising a recognition region at the 3' end that is complementary to the 3' end of the complement of the target sequence sense strand; a nicking enzyme binding site and a nicking site upstream of said recognition region; and a stabilizing region upstream of said nicking site, wherein the portion of the nucleic acid sequence that is complementary to the 3' end of the complement of the target sequence sense strand is 8-15 nucleotides in length; and one or two thermostable nicking enzymes, wherein either one enzyme is capable of nicking at the nicking site of said first and said second templates, or a first enzyme is capable of nicking at the nicking site of said first primer and a second enzyme is capable of nicking at the enzyme site of said second primer.

[0205] In certain embodiments, the target sequence comprises from 1 to 5 nucleotides more than the sum of the nucleotides of said first template recognition region and said second template recognition region. In certain embodiments, the polymerase, nicking enzymes, and templates are in a container. In certain embodiments, the polymerase, nicking enzymes, and templates are in two containers. In other embodiments, the polymerase and nicking enzymes are in a first container, and said templates are in a second container. In some aspects, the polymerase, nicking enzymes, and templates are lyophilized. In some aspects, the kits further comprise instructions for following the method of amplification. The kits may further comprise a cuvette. Or, for example, the kits may further comprise a lateral flow device or dipstick. In some aspects, the lateral flow device or dipstick further comprises a capture probe. In some aspects, the kit further comprises a detector component selected from the group consisting of a fluorescent dye, colloidal gold particles, latex particles, a molecular beacon, and polystyrene beads. In some aspects of the kit, at least one of said templates comprises a spacer, blocking group, or a modified nucleotide.

[0206] Also provided is a kit for amplifying a nucleic acid target sequence, comprising a DNA polymerase; a first template for nucleic acid amplification, comprising a recognition region at the 3' end that is complementary to the 3' end of a target sequence sense strand; a nicking enzyme binding site and a nicking site upstream of said recognition region; and a stabilizing region upstream of said nicking site; a second template for nucleic acid amplification, comprising a recognition region at the 3' end that is complementary to the 3' end of the complement of the target sequence sense strand; a nicking enzyme binding site and a nicking site upstream of said recognition region; and a stabilizing region upstream of said nicking site, wherein said target sequence comprises from 1 to 5 nucleotides more than the sum of the nucleotides of said first template recognition region and said second template recognition region; and one or two thermostable nicking enzymes, wherein either one enzyme is capable of nicking at the nicking site of said first and said

second templates, or a first enzyme is capable of nicking at the nicking site of said first primer and a second enzyme is capable of nicking at the enzyme site of said second primer. In certain aspects of the kit, the portion of the nucleic acid sequence of the first template that is complementary to the 3' end of the target sequence sense strand is 8-15 nucleotides in length, and the portion of the nucleic acid sequence of the second template that is complementary to the 3' end of the complement of the target sequence sense strand is 8-15 nucleotides in length.

[0207] In certain embodiments, the polymerase, nicking enzymes, and templates are in two containers. In other embodiments, the polymerase and nicking enzymes are in a first container, and said templates are in a second container. In some aspects, the polymerase, nicking enzymes, and templates are lyophilized. In some aspects, the kits further comprise instructions for following the method of amplification. The kits may further comprise a cuvette. Or, for example, the kits may further comprise a lateral flow device or dipstick. In some aspects, the lateral flow device or dipstick further comprises a capture probe. In some aspects, the kit further comprises a detector component selected from the group consisting of a fluorescent dye, colloidal gold particles, latex particles, a molecular beacon, and polystyrene beads. In some aspects of the kit, at least one of said templates comprises a spacer, blocking group, or a modified nucleotide.

Examples

Example 1: Sample NEAR™ Amplification Assay

[0208] This example provides an example of a typical DNA wet assay of the present method. Those of ordinary skill in the art understand that numerous modifications may be made to the volumes and format of the reaction, the length of time that the assay is conducted, and the amounts of each reactant.

[0209] Two 96-well microtiter plates are used to set up "wet" assays, a Template/Target plate and a Master Mix plate. To begin, 5 microliters of templates are aliquoted into appropriate wells on the Template Target plate. For the "- target" wells (control wells without target), 5 microliters of dH_2O are added. A reagent master mix is created by combining buffer, salt, dNTPs, enzymes, and dH_2O together in a single tube, using appropriate volumes of each based on the number of samples being tested (see Table within this Example). 40 microliters of reagent master mix is aliquotted into both "-target" and "+ target" (control wells with target) wells of the Master Mix plate, and the plate is sealed with thermal sealant. All of the previous steps were completed in a pre-amplification room with all of the subsequent steps completed in a post-amplification room. The thermal sealant is removed from the Template/Target plate, from only the wells that target will be added to, leaving the "- wells" sealed to avoid potential contamination. 5 microliters of target is aliquoted into the appropriate "+ target" wells. The Template/Target plate is resealed with thermal sealant. Both the Template/Target plate and Master Mix plate are incubated for 2-3 minutes at assay temperature (for example, at 56°C, 57°C, or 58°C, using thermal cyclers. The thermal sealant is removed from both plates. 40 microliters of reagent master mix from the Master Mix plate wells is transferred to the appropriate wells on the Template/Target plate, and the Template/Target plate is resealed with thermal sealant. The samples are incubated for 5-10 minutes at assay temperature. The time for the reaction is calculated from the time that the incubation starts, immediately after the reagent master mix if transferred to the wells on the Template/Target plate, the plate is sealed, and placed in the thermocycler. Reactions are stopped by adding SDS to 0.1% or greater, or by incubating the samples for 2 minutes at 80° C.

[0210] To detect the amplified products, for example, 3-5 microliters of 5 micromolar molecular beacon is added to each well and mixed by pipetting up and down several times. A fluorescence read is performed at the appropriate wavelength based on the fluorophone present on the molecular beacon, at assay temperature, following a 1 minute incubation

[0211] Typical reagent breakdown for single 50 microliter DNA reactions (all volumes in microliters)

Reagent	- Target	+ target	Final Concentration
5XIB2 Buffer	10.0	10.0	1X
100 mM $MgSO_4$	2.5	2.5	10+ 5mM
10 mM dNTPs	1.5	1.5	0.3 mM
8 U/microliter Bst Pol	2.4	2.4	19.2 units
10 U/microliter N. BstNB1	1.50	1.50	15 units
Template 1	2.5	2.5	10-1000 nM
Template 2	2.5	2.5	10-1000 nM
Target	0	5.0	

(continued)

Reagent	- Target	+ target	Final Concentration
H_2O	27.1	22.1	
Total	50.0	50.0	

5X IB2 buffer consists of:
250mM Tris- HCl (pH8.0)
75mM $(NH_4)_2SO_4$
75mM Na_2SO_4
50mM $MgSO_4$
5mM DTT
0.5% Triton X- 100

[0212] A typical reaction does not have a standard target concentration, but target copy per reaction may range from 10-50 at the lower end, for example, to 1E+6 copies in the upper end, for example, or more. In terms of molar concentrations, a 50 microliter assay with 10 copies of target is 3.32e-13 micromolar, where a 50 microliter assay with 50 copies of target is 1.66e-12 micromolar and a 50 microliter assay with 1e6 copies of target is 3.32e-8 micromolar.

[0213] The target sample may consist of, for example, purified DNA or RNA, that has been resuspended in dH_2O or TE, or sample that has not been purified. For example, endocervical swab clinical samples were collected, and sample was eluted and lysed from the swabs using Pierce's Lyse-N-Go PCR reagent (Cat # 78882). Lyse-N-Go is a proprietary formulation that is non-ionic detergent based. Aliquots of each eluted/lysed sample were then added directly to assays, and the results indicate no loss of assay activity. Assays have also been conducted using clinical samples that were collected in viral transport media (VTM), either M4 or M5. The samples collected in VTM were mixed with Pierce's Lyse-N-Go PCR reagent to lyse the target cells, and subsequently aliquots of these samples were added to assays without loss of activity. Finally, the assay has been conducted in the presence of various potential inhibitors, such as sand, soil, clay, urine and serum, and each of these inhibitors was well tolerated.

Example 2: Detection of DNA NEAR ™ assay products by gel electrophoresis

[0214] The amplification reaction products can be visualized by gel electrophoresis. In the absence of target, the templates (with complementary or substantially complementary 3' bases) overlap by one or more bases, polymerase extends in each direction to generate the NEAR™ amplification duplex (Figure 1B); and the amplification proceeds in a similar mechanism to the NEAR™ amplification to amplify a product that is two bases shorter than the target amplified product. In the case of a 25mer assay where the templates end in A and T, the resulting background product is 23 bases. The 27mer assay also forms a 23mer background and 27mer product. Longer reaction products are also amplified. The sequence of these products is hypothesized to be due to the polymerase extension before the nicking enzyme can nick both sides of the NEAR ™ amplification duplex, according to Steps 9B in Figure 1C. Figure 2 shows the NEAR ™ reaction products are easily distinguished from background products by gel electrophoresis.

Example 3: Detection of RNA assay products by gel electrophoresis

[0215] The reaction of the present method can also amplify RNA targets. In this case, the target is Ebola Armored RNA, which is a ~600 base strand of RNA encapsulated by MS2 phage coat proteins to simulate a viral particle. The reaction is designed to amplify a 25-base region of the Ebola genome contained within the encapsulated RNA sequence. Reaction products run on a 20% polyacrylamide gel (Figure 3) show the amplified 25mer product along with 23mer and 20mer background products. This example demonstrates the ability of the reaction to amplify RNA released from virus-like particles.

Example 4: Detection of DNA and RNA assay products by mass spectrometry

[0216] The reaction amplification products of the present methods can also be detected by mass spectrometry using an ESI/TOF system with a front end LC. The reaction products observed are multiple charged ion species. Usually, the -3 or -4 charge state is the major peak in the spectrum (in the range of 1000-3000 AMU), depending on the length of the oligonucleotide product. The sodium adduct is usually present in the spectrum as a peak adjacent to the major peak at roughly 20-25% the intensity. The unique peaks for the positive reactions in the presence of target are visible in both Figures 4 and 5 for the DNA and RNA reactions respectively. The background products formed in these reactions are

not shown in the mass range of these spectra.

Example 5: Real-time detection of the assay amplification

[0217] The amplification reaction of the present method can also be monitored, as shown in Figure 6, in real-time with SYBR II fluorescence. The fluorescence increases as SYBR II intercalates into the amplified double-stranded products. The background products also generate fluorescence at a slower rate than the true product. Optimization of amplification sequence, reaction temperature and reaction buffer conditions are necessary in order to visualize distinct separation between the positive reactions and the negative controls.

Example 6: FRET detection of real-time NEAR ™ assay amplification

[0218] NEAR™ amplification can also be monitored by Fluorescence Resonance Energy Transfer (FRET), as shown in Figure 7. Amplification occurs using dual labeled templates, one on each end (5'- FAM, 3'- BHQ) . Fluorescence is generated from the FAM- labeled oligonucleotide upon cleavage of the template by the nicking enzyme when it becomes double- stranded. Since fluorescence is produced by the initial nicking reaction, this detection method is extremely responsive. Since the 3' ends of the templates are blocked from extension by the quenching label, the production of background fluorescence is inhibited.

Example 7: Molecular beacon detection of real-time NEAR ™ amplification

[0219] A third method of monitoring real-time amplification is using molecular beacons, as shown in Figure 8. In this case, the amplified product hybridizes to the loop region of the molecular beacon resulting in an increase in fluorescence from the separation of the fluorophore and quencher on each end of the hairpin stem. Since this interaction occurs post-amplification, it is considered pseudo-real-time and can be slightly slower in response relative to the FRET approach.

Example 8: False Positive Rate testing

[0220] This experiment was designed to probe the probability that the amplification reaction of the present method will yield a true product in the negative reaction, or a false positive. Reactions directed at specific amplification of a 25mer region specific to the *Bacillus subtilis* genome were run in the presence (n = 120) and absence (n = 320) of *Bacillus subtilis* genomic DNA. End point reactions were run on the mass spectrometer and the area under the curve (AUC) for the product mass peak in the mass spectrum was analyzed. As shown in Figure 9, the results show that none of the 320 negative reactions resulted in a false positive with AUC values equal to the water control. The true positive AUC values were at least 3 standard deviations apart from the true negatives. Overall, these results demonstrate the reproducible nature of the assays of the present methods.

[0221] The Bacillus subtilis assay was developed to target a 25 nucleotide region of the mobA-nprE gene region, with the sequence 5' - TTAACGTCTCTAATTTCAGCTTTTG - 3'. The templates used to amplify this region were, T1 5' - ATGCATGCATGAGTCACATTTAACGTCTCTA - 3', and T2 5' - ATGCATGCATGAGTCACATCAAAAGCTGAAA - 3'. The assay was carried out essentially as described in Example 1, and with the modifications here, for 4 minutes at 56°C with 10,000 copies of Bacillus subtilis genomic DNA plus 100,000 copies of Bacillus thuringiensis genomic DNA (True positives), 10,000 copies of Escherichia coli genomic DNA plus 100,000 Bacillus thuringiensis genomic DNA (True negatives) or no target (water control). Aliquots of each sample were then analyzed by electrospray ionization mass spectrometry to determine the amount of specific product made in each reaction using area under the curve (AUC) calculations.

Example 9: Beacon detection: assay reproducibility with beacon detection

[0222] The molecular beacon detection of reaction products of the present method can also be used as an endpoint reading. As shown in Figure 10, the ratio of reaction products can be manipulated by varying the input ratio of the forward and reverse templates. Skewing the templates to favor one of the reaction products allows the single-stranded product to be available for hybridization to a molecular beacon. The open beacon generates a fluorescent signal. This detection method is extremely reproducible. In this study, two operators performed replicates of the same assay on two different days. The results of this study demonstrate the reproducibility of the assay from one day to the next as well as reproducibility between operators.

Example 10: Assay sensitivity with beacon detection

[0223] The sensitivity of the assay with beacon read-out was tested using a dilution of *Francisella tularensis* genomic DNA. As shown in Figure 11, as few as 50 copies were detected above the no target control.

Example 11: Concentration of amplified products for DNA amplification

[0224] The sensitivity of the assay has also been studied using mass spectrometry detection of the reaction products. Figure 12 shows signal above the no target control down to 100 copies. The data from this study was used to correlate the input copy number to the final amount of amplified product. In this study, the AUC values of the mass spec product peaks were fit to a standard curve to give the estimated final concentration of amplified product for the assay. The amount of amplified product ranges from approximately 250nM to almost 1μM for 1E+2 and 1E+5 copies respectively. This product amount results in a 1E+8 to 7E+10-fold amplification. These reactions were performed without the hot-start conditions, in fact hot-start conditions have been shown to dramatically increase the amount of product amplified, so a further increase in amplification is achieved. The zero copy amplification reaction has a positive final concentration due to the y-intercept value in the standard curve equation.

Example 12: Concentration of amplified products for RNA assay

[0225] A similar study was performed on the amplification of RNA using the present method. A dilution of RNA targets were amplified by the assay of the present method. Products were run on the mass spec and the AUC values of the product peaks were analyzed against a standard curve to determine the concentration of the final product, as shown in Figure 13. A 12 minute amplification starting with 30 and 30,000 copies of initial target results in a 3E+9 to 1E+7 -fold amplification respectively. The lower extent of amplification compared to the DNA amplification could be due to the less efficient reverse transcriptase ability of the polymerase compared to its replication abilities. Also, the RNA:DNA hybrid formed upon the extension of the reverse template is a stronger interaction compared to a normal DNA:DNA hybrid and will have less breathing to allow for the forward or another reverse template to displace one strand. However, amplification products from the RNA reaction were detected down to <100 copies.

Example 13: NEAR ™ reaction specificity for DNA

[0226] Since the reaction products are usually between 20 and 30 bases in length, the question arises as to whether or not these short amplification assays can be specific enough to target a single sequence region with other near neighbor genomes present. The reaction was tested for its specificity by running the amplification reaction in the presence and absence of varying amounts of the near neighbor genomic DNA (Figure 14). In this case, the assay detects a specific sequence in the pXO2 plasmid of *Bacillus anthracis* and the near neighbor genome is *Bacillus thuringiensis* (kurstaki). The reactions were analyzed by the AUC values for the product peaks. The figure below demonstrates that in the absence of the correct target (*Bacillus anthracis*), there is no true product amplified (the levels are so low that they are not visible on the scale of the graph). The amount of amplification of the positive reactions is consistent, with larger error bars for the 0 and 5E+5 copies of *Bacillus thuringiensis* (5E+4 copies of *Bacillus anthracis*) due to a single lower value for one of the triplicate runs. Overall the experiment demonstrates that the reaction is very specific to the target sequence when the assay is designed within a unique region of the genome.

Example 14: Interferent testing

[0227] A panel of interferents was tested to monitor the effect of each on amplification. Figure 15 demonstrates the robust nature of the assay of the present methodin the presence of interferents. Some interferents that are known to inhibit PCR, such as humic acid, did not appear to inhibit the assay, though the amount of each interferent is unknown. From statistical analysis only interferent B, C, and E were statistically different from the control assay x. In the B, C, and E cases, the difference resulted in increased product amplification.

Example 15: Multiplexing of two sequences with DNA assays

[0228] A DNA duplex was designed for capillary electrophoresis (CE) detection. Amplification products were 25 bases (*Bacillus anthracis* assay, *Ba*) and 27 bases (*Bacillus subtilis* assay, *Bs*) in length with background production of a 23mer. The reaction was run for 10 minutes in the presence or absence of 5E+5 copies of the respective genomic DNA target. The samples were run on a 20% polyacrylamide gel to visualize the reaction products. Figure 16 indicates the presence of positive product amplification when *Bacillus subtilis* only is present as well as when both *Bacillus subtilis* and *Bacillus*

anthracis are present.

Example 16: DNA assay duplex specificity

5 **[0229]** The DNA duplex reaction with *Bacillus subtilis* (*Bs*) and *Bacillus anthracis* (*Ba*) was shown to be specific to the respective genomes. The assays were run in the presence of the near neighbor, *Bacillus thuringiensis*, as shown in Figure 17. In the negative reaction where both template sets are present as well as the *Bacillus thuringiensis* genomic DNA, there is no product band in the 25 or 27mer region. Product bands appear only when the specific genomic target is present, which demonstrates the specificity of the duplex reaction.

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Example 17: Multiplexing with RNA assays

[0230] An MS2 assay that amplifies a 27mer product and an Ebola assay that amplifies a 25mer product was developed and multiplexed so that all templates are present in each assay and amplification of products is dependent on the target 15 present. This combination of templates forms background products that are 23 bases and 20 bases in length. The gel shown in Figure 18 demonstrates the ability for the reaction of the present method to amplify multiple RNA targets in a single reaction.

Example 18: Amplification from lysed spores

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[0231] Amplification was performed on semi-processed samples to determine whether it is possible to amplify DNA released from spores through lysis. The negative control reaction contained DNase-treated spores, unlysed, so no DNA should be present to amplify. The positive control reaction contained purified genomic DNA at concentrations around the amount of DNA estimated to be released through lysis. Results in Figure 19 show that amplification with unlysed 25 DNase-treated spores results in no product amplification as expected, whereas the three samples lysed before amplification resulted in product amounts in the range of the theoretical amounts.

Example 19: Capture and Extension

30 **[0232]** The reaction products of the present method can also be detected on a solid surface. A capture probe attached at the 5' end to the surface through a biotin/streptavidin attachment can bind to the reaction products from which polymerase extends to form a stable duplex that SYBR and any intercalating dye can detect. The capture probe is designed to favor extension through binding to the true product over background products because the 3' base of the capture probe is complementary to the middle spacer base in the product which is not present in either of the templates or the background 35 products. Figure 20 demonstrates the increased fluorescence of the products in the presence of the capture probe and polymerase over the average binding (same reaction in the absence of polymerase, to preclude extension of the capture probe) and the no target control where only background products are amplified, but cannot form a stable duplex with the capture probe for polymerase to extend.

40 **Example 20:** Surface NEAR ™ FRET DNA Assay

[0233] The reaction of the present method can also be performed with the templates immobilized on the surface. The templates for FRET detection of surface amplification usually have three modifications: one 5' biotin with a TEG spacer, one FAM fluorophore internal to the biotin, and a quencher on the 3' end which serves to block background amplification 45 as well as to quench the FAM fluorophore. The template is immobilized on the surface through biotin/streptavidin attachment. Figure 21 demonstrates that with both templates immobilized along with additional mixing, the reaction proceeds at a much slower rate than the solution amplification rate (amplification in 16 minutes for 1E+6 copies of genomic DNA). When a single template is immobilized on the surface and the other template is free in solution, the amplification reaction is increased to 10 minute detection for 1E+6 copies of genomic DNA. Fluorescence from background products 50 is observed ~3.5 minutes after the product signal, similar to what is observed for solution phase kinetics, but slowed considerably.

Example 21: Healthcare Example

55 *Chlamydia trachomatis* (Ct) Assay

[0234] An assay of the present method was performed to detect the presence of a Chlamydia trachomatis (Ct) target sequence. A 2- fold dilution series of synthetic DNA containing the target sequence for the Ct P2_2 assay was used to

determine the limit of detection of the assay. The reaction was carried out essentially as described in Example 1, with some modifications as described in this example. The dilution series started with 10, 000 copies of target DNA, and proceeded to less than 1 copy per reaction. A 'no target' control sample was also included in this experiment. Reactions were performed on a 96- well microtiter plate in 50 microliter volumes in the following buffer: 50 mM Tris- HCl, pH 8.0, 30 mM NaCl, 15 mM $(NH_4)_2$ (SO_2), 15 mM Mg_2SO_4, 1 mM DTT, 0.1% Triton X- 100 with 0.3 mM dNTPs, 19.2 units of Bst DNA polymerase and 15 units of Nt.BstNBI nicking enzyme. Templates were added at a ratio of 200 nM : 100 nM (Template 1: Template 2) . Reactions were performed as follows: On plate 1, 5 microliters of template mix was added to each well in a pre- amplification room, and sealed. On plate 2, 40 microliters of master mix was added to each well in a pre- amplification room, and sealed. The master mix consisted of dH_2O plus all assay components listed above, except templates. The two plates were then transferred to a post- amplification room where 5 microliters of target was added to each well of plate 1 (excluding the 'no target' control wells) . The two plates were then transferred to thermal cyclers pre- heated to 56°C for 2- 3 minute pre- incubations at 56°C. The contents of plate 2 were then transferred to plate 1 which was then incubated for 5 minutes at 56°C (amplification step) . Following this incubation, the reactions were stopped by inactivating the enzymes at 80°C for 2 minutes. Subsequently, a molecular beacon specific for the amplified Ct P2_2 product was added to a final concentration of 300 nM and fluorescence was detected at 56°C. All samples were performed in triplicate, with error bars showing standard deviations.

[0235] The Ct P2_2 assay was performed using two templates, template 1 (5'- ATGCATGCATGAGTCACATAGGCT-TATGGAG- 3') and template 2 (5'- ATGCATGCATGAGTCACATTTATACCGCTTA- 3') at a 200 nM: 100 nM final template concentration. The molecular beacon used for fluorescence detection, MB 5.18, contained a 5'- FAM fluorophore and 3'- BHQ1 quencher, with the following sequence: 5'- ctggcTACCGCTTAACTCCATAAgccag- 3'.

[0236] The results are shown in Figure 22, and show that the assay can efficiently detect less than 10 copies of target in a sample. Figure 22B shows that even about 1-2 copies can be detected, but because of the dilution experiment, some wells may, statistically, not have any target DNA (compare Fig. 22b, bars 1.2 a, b, and c).

The target sequence for the Ct P2_2 assay is 5'AGGCTTATGGAGTTAAGCGGTATAA - 3'. Clinical samples, such as those collected on endocervical or vaginal swabs, or those collected on swabs and then transferred to viral transport media such as M4 or M5 can be prepared for use in anassay as follows. Each swab is placed into a 1.5 milliliter or 2.0 milliliter eppendorf tube containing 300 microliters to 1 milliliter of Pierce's Lyse-N-Go PCR reagent (Cat # 78882). The mixture is allowed to incubate at room temperature for 5 - 10 minutes, with occasional mixing. An aliquot of the eluted and lysed sample is then added directly to an assay. For samples present in viral transport media, an aliquot of the sample can be transferred to an eppendorf tube containing an equal or greater volume of Pierce's Lyse-N-Go PCR reagent (at a sample:Lyse-N-Go ratio of 1:1, 1:2, 1:10, 1:20, etc....) and allowed to incubate at room temperature for 5 - 10 minutes, with occasional mixing. An aliquot of the eluted and lysed sample is then added directly to an assay.

Example 22 Food Safety Applications

Listeria monocytogenes assay

[0237] To demonstrate the effectiveness of the assay of the present method for the specific detection of a food pathogen, assays were conducted on *Listeria monocytogenes,* one of the most significant threats to food safety from ready-to-eat food products. The assays were performed essentially as described in Example 1, with modifications described in this Example. *L. monocytogenes* strain EGD-e genomic DNA was assayed with increasing amounts of genomic DNA from the closely related non-pathogenic species *L. innocua* strain Clip11262. As shown in Figure 23, Negative control reactions with no DNA present showed only background levels of fluorescence, and the increasing amounts of *L. innocua* DNA up to 1 million genome equivalents per 50 microliter reaction showed no significant increase in the background fluorescence. However, the addition of 1,000 genome equivalents of *L. monocytogenes* was easily detected with a substantial increase in fluorescence, and was unaffected by the presence of the *L. innocua*, even when the non-pathogenic *L. innocua* was present in 1000-fold excess, which was 1 million genome equivalents per 50 microliter reaction. Each reaction consisted of: 46 mM Tris buffer pH 8.5; 50 mM NaCl; 10 mM KCl; 10 mM $(NH_4)_2SO_4$; 5 mM $MgCl_2$; 10 mM $MgSO_4$; 0.5 mM dithiothreitol; 0.1% Triton X-100; 0.01 mM EDTA; 0.3 mM each dATP, dCTP, dGTP, and dTTP; 19.2 units *Bst* DNA polymerase from New England Biolabs, Inc.; 15 units Nt.BstNBI nicking endonuclease from New England Biolabs, Inc.; 200 nM of the first oligonucleotide; and 2 micromolar of the second oligonucleotide. The oligonucleotides and *Listeria* genomic DNA were incubated separately from the enzyme buffer mixture at 56 °C, and then 5 microliters of this mixture was added to 45 microliters of enzyme buffer mixture. The reaction was incubated at 56 °C for 10 minutes, and then 80 °C for two minutes. After this, 3.2 microliters of a 5 μM solution of a Molecular Beacon was added to each reaction. The sequence of the Molecular Beacon was specific for the amplified *L. monocytogenes* sequence with a fluorophore and quencher on the 5' and 3' ends, respectively. Following the addition of the Molecular Beacons, the reactions were incubated at 56 °C for one minute, and then fluorescence measurements were made. Each assay condition was tested in duplicate, and the average fluorescence values are shown. The target se-

quence for the Listeria monocytogenes assay is 5' - AAAGCAAGAGAAAGTTATCGTGTAT - 3'. The template sequences are as follow: T1 5' - ATGCATGCATGAGTCACATAAAGCAAGAGAA - 3'and T2 5' - ATGCATGCATGAGTCACATAT-ACACGATAAC - 3'.

5 **Example 23:** Viral RNA Example

[0238] A 10- fold dilution series of purified viral RNA from a viral positive clinical sample was used to determine the limit of detection of the assay. The viral RNA was purified using a commercially available viral RNA purification kit. A 'no target' negative control sample was included. Reactions were performed on 96- well microtiter plates in 50 microliter
10 volumes in the following buffer: 50 mM Tris- HCl, pH 8.0, 30 mM NaCl, 15 mM $(NH_4)_2$ (SO_2), 10 mM Mg_2SO_4, 1 mM DTT, 0.1% Triton X- 100 with 0.1 mM dNTPs, 19.2 units of Bst DNA polymerase, 7.5 units of Nt.BstNBI nicking enzyme and 4 units of OmniScript reverse transcriptase. Templates were added at a ratio of 400 nM : 20 nM (Template 1: Template 2) . Reactions were performed as follows: On plate 1, 5 microliters of template mix was added to each well in a pre- amplification room, and sealed. On plate 2, 40 microliters of master mix was added to each well in a pre- amplification
15 room, and sealed. The master mix consisted of water plus all assay components listed above, except templates. The two plates were then transferred to a post- amplification room where 5 microliters of target was added to each well of plate 1 (excluding the 'no target' control wells) . The two plates were then transferred to thermal cyclers pre- heated to 56°C for 2- 3 minute pre- incubations at 56°C. The contents of plate 2 were then transferred to plate 1 which was then incubated for 5 minutes at 56°C (amplification step) . Following this incubation, the reactions were stopped by inactivating
20 the enzymes at 80°C for 2 minutes. Subsequently, molecular beacon specific for the amplified product was added to a final concentration of 300 nM and fluorescence was detected at 56°C. All samples were performed in triplicate, with error bars showing standard deviations. Results are shown in Figure 24.

[0239] The viral RNA assay was performed using two templates (template 1: 31 nucleotides long, and template 2: 31 nucleotides long) at a 400 nM:20 nM final template concentration. The molecular beacon used for fluorescence detection
25 (MB), contained a 5'-FAM fluorophore and 3'-BHQ1 quencher, with a 29 nucleotide long sequence. The length of the target sequence was 26 nucleotides.

Example 24: Agriculture Application: Detection of Genetically Modified Traits in Crops Assay Sample Preparation for Genetically Modified (GMO) and Conventional (non-GMO) Maize:

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[0240] The assay of the present methods may be used to detect genetically modified organisms (GMO) in agricultural applications. Theassay was used to detect the presence of the *bar* gene, inserted into the maize genome, in a background of unmodified maize DNA. The *bar* gene confers resistance to the broad-spectrum herbicide glufosinate. The assays were conducted essentially as described in Example 1, with modifications as described herein. Genetically modified and
35 conventional (unmodified) maize seeds were ground to an appropriate level of coarseness, and nucleic acids were extracted using a standard buffer. The extracted material was purified using a size-exclusion column according to the manufacturer's instructions. Purified nucleic acids were combined to yield a final concentration of 5% *bar*-modified maize in a conventional background (*e.g.*, 5 microliters of *bar* maize DNA extract combined with 95 microliters conventional maize DNA extract), or used unmixed in the case of 100% conventional maize. The oligonucleotide sequences used to
40 detect the bar gene are listed below.

Template 1: ATGCATGCATGAGTCACATCATCGTCAACCA
Template 2: ATGCATGCATGAGTCACATTGTCTCGATGTA

45 **[0241]** The templates were designed to produce the following products:

Product 1: CATCGTCAACCACTACATCGAGACA
Product 2: TGTCTCGATGTAGTGGTTGACGATG

50 **[0242]** The assay reagents used were: 9.6 units of Bst. Polymerase (NEB), 15 units of N.BstNBI nicking enzyme (NEB), 5 microliters Thermopol I Buffer (NEB), 2.5 microliters NEB Buffer 3, 12 mM $MgSO_4$, 0.3 mM dNTPs, 2.5% DMSO (dimethyl sulfoxide), 5 microliters sample, templates and water. The oligonucleotides were present at initial concentrations of 10 nM (Template 1) and 100 nM (Template 2). Water was used to adjust the final volume to 50 microliters, and a 10 minute assay was performed at 56° C, followed by a 2 minute incubation at 94° C to inactivate the enzymes, followed
55 by detection at 56° C with a specific molecular beacon at a final concentration of 300 nM. The sequence of this molecular beacon is:

5' FAM- CCTCGCCGTCAACCACTACATCGAGCGAGG- BHQ1- 3'.

The results are shown in Figure 25.

Example 25: Detection of MicroRNA (miRNA)

5 Assay Sample Preparation for microRNAs from MDA-MB-231 Human Breast Cancer Cells:

[0243] MDA- MB- 231 Human breast cancer cells (ATCC number HTB- 26) are known to express elevated levels of microRNA- 21 (Iorio, M.V. et al., 2005. MicroRNA gene expression deregulation in human breast cancer. Cancer Res. 65: 7065- 7070) . An assay for miR- 21 was developed that detects the mature microRNA- 21 sequence:

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5'UAGCUUAUCAGACUGAUGUUGA3'

[0244] The template sequences used were (nicking enzyme sequences are underlined):

15 Template 1: ATGCATGCATGAGTCACATTAGCTTATCA
Template 2: ATGCATGCATGAGTCACATTCAACATCAG

[0245] The templates were designed to produce the following products:

20 Product 1: TAGCTTATCAGACTGATGTTGA
Product 2: TCAACATCAGTCTGATAAGCTA

[0246] The assay was conducted essentially as described in Example 1, with modifications described herein. To obtain RNA, MDA-MB-231 cells were propagated and sub-cultured, using standard methods familiar to those skilled in the art,
25 in Dulbecco's Modified Eagle's Medium (Invitrogen) supplemented with 10% fetal bovine serum, glucose and antibiotics. Prior to reaching confluency, cells were removed from the plate by treatment with trypsin, and subsequently washed in phosphate buffered saline prior to freezing at -80° C. Cells were later defrosted and a portion used for RNA isolation with TRI Reagent (Molecular Research Center, Inc.) according to the manufacturer's instructions. Purified RNA was quantified using UV absorbance at 260 nm.
30 **[0247]** According to the Molecular Research Center TRI Reagent manual, 1 ng of purified RNA corresponds to approximately 100 cells of starting material. Various amounts of purified RNA were used in an assay comprised of the following reagents: 50 mM Tris-HCl, pH 8.0, 30 mM $(NH_4)_2SO_4$, 30 mM Na_2SO_4, 1 mM DTT, 0.1% Triton X-100, 10 mM $MgSO_4$, 0.1 mM dNTPs, 19.2 units of Bst. Polymerase (New England Biolabs), 7.5 units of N.BstNBI nicking enzyme (New England Biolabs), 7.4 units Omniscript Reverse Transcriptase (Qiagen), two oligonucleotides at 100 nM each,
35 sample and water. Water was used to adjust the final volume to 50 microliters, and a 20 minute assay was performed at 56° C, followed by a 2 minute incubation at 94° C to inactivate enzymes. The product was measured using electrospray ionization mass spectrometry, and product amounts were quantified by calculating the area under the curve. The results of the assay are shown in Figure 26.

40 **Example 26:** Detection of a Genomic DNA Target

[0248] An assay of the present method was performed essentially as described in Example 1, using oligo templates designed to bind to a genomic target. Dilution experiments were conducted to determine the lower limit of detection. As shown in Figure 27, there was consistent detection at 50 genome copies. When the diluted sample contained 10 genomic
45 copies, there was detection, however, statistically, the detection was not as consistent.
[0249] Example 26 (Figure 27) depicts a Neisseria gonorrhoeae assay. The assay targets the pilQ gene, specifically the sequence 5' - ACTCTACCAACACGGAACTCAAAAA - 3'. The template sequences used to amplify this target were: T1 5' - ATGCATGCATGAGTCACATTTTTTGAGTTCC - 3', and T2 5' - ATGCATGCATGAGTCACATACTCTACCAACA - 3'. The assay was carried out essentially as described in Example 1, with the modifications herein. Briefly, the assay
50 was performed for 5 minutes at 56°C followed by a heat inactivation step at 80°C for 2 minutes to stop the reaction. End point detection of amplified specific product was performed using 300 nanomolar of a molecular beacon containing a 5'-fluorophore and 3'-quencher that was specific to the amplified specific following a 1 minute incubation at 56°C. The molecular beacon sequence was: 5' - CGCATGGAGTTCCGTGTTGGTAGACATGCG - 3'.
[0250] **Example 27:** Calculation of Specific Product Generated in a B. subtilisAssay Anassay of the present method
55 was performed essentially as described in Example 1, using oligo templates designed to bind to a Bacillis subtilis target sequence, the target was the ppsA gene:

Target sequence (25mer) 5' - CCAAGCTCAAAAAAGGAATCGTGAA - 3'

T1 5' - ATGCATGCATGAGTCACATCCAAGCTCAAAA - 3'
T2 5' - ATGCATGCATGAGTCACATTTCACGATTCCT - 3'

[0251] As shown in Figure 28, the linear regression showed an excellent correlation between the amount of the reference oligo added to a sample and area under the curve (AUC). This equation was used to determine the amount of specific product generated when 50 or 500 copies of genomic DNA target were added to a reaction. The reaction was performed for 5 minutes. The fold amplification was calculated and is presented in the Table below.

Table 5

Specific product 1944 yields (x=y-b/m)				
Sample	**AUC signal**	**Product (nM)**	**Product (50 microliter reaction, in pmoles)**	**Fold amplification,**
50-1	1394	2851	0.1426	1.72E+09
50-2	1495	3049	0.1525	1.84E+09
50-3	1175	2421	0.1211	1.46E+09
50-4	1072	2219	0.1110	1.34E+09
500-1	1799	3645	0.1823	2.20E+08
500-2	1837	3720	0.1860	2.24E+08
500-3	1472	3004	0.1502	1.81E+08
500-4	1438	2937	0.1469	1.77E+08

[0252] Calculations were based on the following: B. subtilis genome = 4214814 nucleotides, molecular weight (g/mole) of 2781777240. Avogadro's number (molecules/mole) = 6.02E+23. For 50 genome copies in moles, this results in 8.30E-23, for 500 genome copies in moles, this results in 8.30E-22.

Example 28: Effect of Different Spacer Lengths

[0253] A series of *Chlamydia trachomatis* (Ct) assays was performed essentially as describe in Examples 1 and 21, using various templates as shown in Figures 29 and 30. Figure 29 shows the results of the reaction, Figure 30 provides more detail as to the template design. The reaction was conducted for 10 minutes using either 0 or 100 copies of target. A series of oligonucleotide templates was prepared, with spacer region lengths (number of nucleotides on the target sequence between the binding sites of the oligo templates, if the templates were bound) ranging from 1 to 11. Optimal spacer lengths for this experiment were 1, 2, 3, or 4.

[0254] A similar set of experiments was conducted for a viral RNA target, following essentially the same methods as those described in Example 23m using spacer lengths of 2, 5, 6, 7, and 8. As determined by mass spectrometry, optimal specific product detection was found using spacer lengths of 2 and 5, and no specific product was detected in this assay where the spacer length was 6 or greater and the reaction was run for 20 minutes.

[0255] Similar experiments were also conducted with other targets. For some targets, such as miR-21, when no spacer nucleotides were included in the template design, product was detected whether or not a target sequence was present in the reaction. Product was detected whether or not target DNA was present in the assay, indicating that the template set was producing the specific product without a need for the target being present. In other experiments, a spacer region of 0 nucleotides did result in specific product. Therefore, in designing templates for the assays discussed herein, more than one set of templates should be prepared, to determine the length of the spacer region that is optimal to produce specific product from a particular target.

Example 29: Effect of the Stabilizing Regions

[0256] A set of *Chlamydia trachomatis* (Ct) assays was performed essentially as described in Examples 1 and 21. Templates were prepared that either included, or did not include, the stabilizing region (5'ATGCATGCAT). The reaction was performed for 10 minutes, with either 0 or 100 copies of target DNA. Analysis was performed using real-time SybrGreen fluorescence detection. As shown in Figure 31, the samples containing templates without stabilizing regions showed no amplification. In another set of assays, using viral RNA, either 0 or 1000 copies of target was included in the assay. The samples containing templates without stabilizing regions showed no amplification, while those with stabilizing regions showed rapid amplification.

Example 30: Effect of Mg^{+2} Concentration

[0257] A set of *Chlamydia trachomatis* (Ct) assays was performed essentially as described in Examples 1 and 21. The assays were conducted using varying concentrations of Mg^{+2}. As shown in Figure 32, for this set of assays, a complete loss of activity was found when 6 mM Mg^{+2} was present, and a significant drop in activity was found when 9 mM Mg^{+2} was present. At concentrations from 12 mM to 21 mM Mg^{+2} , the assay performed optimally.

Example 31: Examples of other Template/Target Combinations

[0258] The present methods are not limited to the specific templates and targets provided in the present embodiments and examples. Other targets and templates may be used to perform the isothermal amplification methods discussed herein. Examples of other targets and templates include, but are not limited to, those presented in Figure 34. Those of ordinary skill in the art recognize that other templates may be designed for the targets presented in the Figure, related target sequences to those presented in the Figure may be used in the reaction, and target sequences not included in the Figure are within the scope of the present methods.

[0259] The entirety of each patent, patent application, publication and document referenced herein hereby is incorporated by reference. Citation of the above patents, patent applications, publications and documents is not an admission that any of the foregoing is pertinent prior art, nor does it constitute any admission as to the contents or date of these publications or documents.

[0260] Singular forms "a", "an", and "the" include plural reference unless the context clearly dictates otherwise. Thus, for example, reference to "a subset" includes a plurality of such subsets, reference to "a nucleic acid" includes one or more nucleic acids and equivalents thereof known to those skilled in the art, and so forth. The term "or" is not meant to be exclusive to one or the terms it designates. For example, as it is used in a phrase of the structure "A or B" may denote A alone, B alone, or both A and B.

[0261] Unless defined otherwise, all technical and scientific terms used herein have the same meanings as commonly understood by one of ordinary skill in the art to which this invention belongs. Although any methods and systems similar or equivalent to those described herein can be used in the practice or testing of the present invention, the methods, devices, and materials are now described. All publications mentioned herein are incorporated herein by reference for the purpose of describing and disclosing the processes, systems, and methodologies that are reported in the publications which might be used in connection with the invention. Nothing herein is to be construed as an admission that the invention is not entitled to antedate such disclosure by virtue of prior invention.

[0262] Modifications may be made to the foregoing without departing from the basic aspects of the invention. Although the invention has been described in substantial detail with reference to one or more specific embodiments, those of ordinary skill in the art will recognize that changes may be made to the embodiments specifically disclosed in this application, and yet these modifications and improvements are within the scope and spirit of the invention. The invention illustratively described herein suitably may be practiced in the absence of any element(s) not specifically disclosed herein. Thus, for example, in each instance herein any of the terms "comprising", "consisting essentially of, and "consisting of may be replaced with either of the other two terms. Thus, the terms and expressions which have been employed are used as terms of description and not of limitation, equivalents of the features shown and described, or portions thereof, are not excluded, and it is recognized that various modifications are possible within the scope of the invention. Embodiments of the invention are set forth in the following claims.

[0263] Paragraphs of the invention:

1. A method for nucleotide sequence amplification, which comprises:

    combining a target nucleic acid having a target nucleotide sequence with (i) a polymerase, (ii) a first template nucleic acid that hybridizes to the a first strand of the target nucleotide sequence, and (iii) a second template nucleic acid that hybridizes to the complement of the first strand of the target nucleotide sequence, in an amplification reaction,

    under conditions in which the polymerase extends the template nucleic acids, thereby generating extended template nucleic acid amplicons; wherein:

        the target nucleotide sequence is between 20 and 40 nucleotides in length;
        the target nucleotide sequence is amplified 1E+6-fold or more in about ten minutes; and
        the foregoing steps are conducted under substantially isothermal conditions.

2. A method for nucleotide sequence amplification, which comprises:

combining a target nucleic acid having a target nucleotide sequence with (i) a polymerase, (ii) a first template nucleic acid that hybridizes to the a first strand of the target nucleotide sequence, and (iii) a second template nucleic acid that hybridizes to the complement of the first strand of the target nucleotide sequence, in an amplification reaction,
5 under conditions in which the polymerase extends the template nucleic acids,
thereby generating extended template nucleic acid amplicons; wherein:

the target nucleotide sequence is between 20 and 40 nucleotides in length;
the first template comprises a nucleic acid sequence comprising a first template recognition region at the
10 3' end that is complementary or substantially complementary to the 3' end of the first strand of the target nucleotide sequence;
the second template comprises a nucleotide sequence comprising a second template recognition region at the 3' end that is complementary or substantially complementary to the 3' end of the complement of the first strand of the target nucleotide sequence;
15 the target nucleotide sequence comprises from 1 to 5 nucleotides more than the sum of the nucleotides of the first template recognition region and the second template recognition region;
the target nucleotide sequence is amplified 1E+6-fold or more in about ten minutes; and the foregoing steps are conducted under substantially isothermal conditions.

20 3. A method for nucleotide sequence amplification, which comprises:

combining a target nucleic acid having a target nucleotide sequence with (i) a polymerase, (ii) a first template nucleic acid that hybridizes to the a first strand of the target nucleotide sequence, and (iii) a second template nucleic acid that hybridizes to the complement of the first strand of the target nucleotide sequence, in an
25 amplification reaction,
under conditions in which the polymerase extends the template nucleic acids,
thereby generating extended template nucleic acid amplicons; wherein:

the first template comprises a nucleic acid sequence comprising a first template recognition region at the
30 3' end that is complementary or substantially complementary to the 3' end of the first strand of the target nucleotide sequence,
wherein the recognition region is 8-15 nucleotides long;
the second template comprises a nucleotide sequence comprising a second template recognition region at the 3' end that is complementary or substantially complementary to the 3' end of the complement of the
35 first strand of the target nucleotide sequence, wherein the recognition region is 8-15 nucleotides long;
the target nucleotide sequence is amplified 1E+6-fold or more in about ten minutes; and the foregoing steps are conducted under substantially isothermal conditions.

4. The method of paragraph 1, wherein
40 the first template comprises a nucleic acid sequence comprising a first template recognition region at the 3' end that is complementary or substantially complementary to the 3' end of the first strand of the target nucleotide sequence; and
the second template comprises a nucleotide sequence comprising a second template recognition region at the 3' end that is complementary or substantially complementary to the 3' end of the complement of the first strand of the
45 target nucleotide sequence.
5. The method of paragraph 3, wherein the target nucleotide sequence comprises from 1 to 5 nucleotides more than the sum of the nucleotides of the first template recognition region and the second template recognition region.
6. The method of any of paragraphs 2, 3, 4, or 5, wherein the first template and second templates comprise nicking enzyme binding sites and nicking sites upstream of the recognition regions, and the amplification reaction further
50 comprises one or more nicking enzymes that are capable of nicking at the nicking site of said forward and said reverse templates, wherein either one nicking enzyme is capable of nicking both of said templates, or each template is capable of being nicked by at least one of the nicking enzymes, and wherein said one or more nicking enzymes do not nick within said target sequence.
7. The method of paragraph 6, wherein the target nucleotide sequence comprises 1 nucleotide more than the sum
55 of the nucleotides of the first template recognition region and the second template recognition region.
8. The method of paragraph 6, wherein the target nucleotide sequence comprises 2 nucleotides more than the sum of the nucleotides of the first template recognition region and the second template recognition region.
9. The method of paragraph 6, wherein the target nucleotide sequence comprises 3 nucleotides more than the sum

of the nucleotides of the first template recognition region and the second template recognition region.
10. The method of any of paragraphs 1-9, wherein the target nucleic acid is double stranded or single stranded.
11. The method of paragraph 10, wherein the target nucleic acid is double-stranded DNA.
12. The method of paragraph 10, wherein the target nucleic acid is single-stranded DNA.
5 13. The method of paragraph 10, wherein the target nucleic acid is RNA
14. The method of paragraph 11, wherein the target nucleic acid is selected from the group consisting of genomic DNA, plasmid DNA, viral DNA, mitochondrial DNA, and synthetic double-stranded DNA.
15. The method of paragraph 12, wherein the target nucleic acid is selected from the group consisting of viral DNA, cDNA, and synthetic single-stranded DNA.
10 16. The method of paragraph 13, wherein the target nucleic acid is selected from the group consisting of messenger RNA, viral RNA, ribosomal RNA, transfer RNA, micro RNA, micro RNA precursor, and synthetic RNA.
17. The method of any of paragraphs 1-16, wherein said DNA polymerase is a thermophilic polymerase.
18. The method of any of paragraphs 1-16, wherein said polymerase is selected from the group consisting of Bst (large fragment), 9°N, $Vent_R$® (exo-) DNA Polymerase, Therminator, and Therminator II.
15 19. The method of any of paragraphs 1-19, wherein said polymerase is Bst (large fragment).
20. The method of any of paragraphs 6-12, wherein the first and second templates comprise nicking enzyme binding sites recognized by the same nicking enzyme and said first and said second nicking enzyme are the same.
21. The method of paragraph 19, wherein said nicking enzymes are selected from the group consisting of Nt.BspQI, Nb.BbvCi, Nb.BsmI, Nb.BsrDI, Nb.BtsI, Nt.AlwI, Nt.BbvCI, Nt.BstNBI, Nt.CviPII, Nb.Bpu10I, and NtBpu10I.
20 22. The method of any of paragraphs 1-20, wherein the portion of the nucleic acid sequence of the first strand that is complementary or substantially complementary to the first strand of the target nucleotide sequence is 8-15 nucleotides in length and wherein the portion of the second strand that is complementary or substantially complementary to the target nucleotide sequence is 8-15 nucleotides in length.
23. The method of any of paragraphs 1-22, wherein the first template is provided at the same concentration as the
25 second template.
24. The method of any of paragraphs 1-22, wherein one of the first or second templates is provided at a ratio to the other template at the range of ratios of 1:100 to 100:1
25. The method of any of paragraphs 1-25, further comprising a second polymerase.
26. The method of paragraph 25, wherein at least one of the first or second polymerases comprises reverse tran-
30 scriptase activity.
27. The method of any of paragaphs 1-26, wherein the amplification is conducted between 54°C and 60°C.
28. The method of any of paragraphs 1-26, wherein the amplification is conducted between 56°C and 58°C.
29. The method of any of paragraphs 1-28, wherein the amplification reaction is held at a constant temperature for 1 to 10 minutes.
35 30. The method of any of paragraphs 1-28, wherein the amplification reaction is held at a constant temperature for 1 to 20 minutes.
31. The method of any of paragraphs 1-30, further comprising detecting the amplification product.
32. The method of paragraph 31, wherein said amplification product is detected by detection method selected from the group consisting of gel electrophoresis, mass spectrometry, SYBR I fluorescence, SYBR II fluorescence, SYBR
40 Gold, Pico Green, TOTO-3, intercalating dye detection, fluorescence resonance energy transfer (FRET), molecular beacon detection, surface capture, capillary electrophoresis, incorporation of labeled nucleotides to allow detection by capture, fluorescence polarization, and lateral flow capture.
33. The method of any of paragraphs 1-32, wherein at least two target sequences are capable of being amplified.
34. The method of any of paragraphs 1-33, wherein said amplification products are detected on a solid surface.
45 35. The method of any of paragraphs 1-34, wherein at least one capture probe is immobilized on a solid surface.
36. The method of any of paragraphs 1-35, wherein at least one of said templates comprises a spacer, blocking group, or a modified nucleotide.
37. The method of any of paragraphs 1-36, wherein the target nucleotide sequence is amplified 1E+6-fold or more in about five minutes.
50 38. The method of any of paragraphs 1-36, wherein the target nucleotide sequence is amplified 1E+6-fold or more in about 2.5 minutes.
39. The method of any of paragraphs 1-36, wherein the target nucleotide sequence is amplified 1E+7-fold or more in about five minutes.
40. The method of any of paragraphs 1-36, wherein the target nucleotide sequence is amplified 1E+8-fold or more
55 in about five minutes.
41. The method of any of paragraphs 1-36, wherein the target nucleotide sequence is amplified 1E+9-fold or more in about five minutes.
42. A method for amplifying a double-stranded nucleic acid target sequence, comprising

a) contacting a target DNA molecule comprising a double-stranded target sequence, having a sense strand and an antisense strand, with a forward template and a reverse template, wherein

i) said forward template comprises a nucleic acid sequence comprising a recognition region at the 3' end that is complementary to the 3' end of the target sequence antisense strand; a nicking enzyme binding site and a nicking site upstream of said recognition region and a stabilizing region upstream of said nicking site, wherein the portion of the nucleic acid sequence that is complementary to the 3' end of the target antisense strand is 8-15 nucleotides in length;
ii) said reverse template comprises a nucleotide sequence comprising recognition region at the 3' end that is complementary to the 3' end of the target sequence sense strand, a nicking enzyme binding site and a nicking site upstream of said recognition region, and a stabilizing region upstream of said nicking site, wherein the portion of the nucleic acid sequence that is complementary to the 3' end of the target antisense strand is 8-15 nucleotides in length;

b) providing a first nicking enzyme that is capable of nicking upstream, downstream, or at the nicking site of said forward template, and does not nick within said target sequence;
c) providing a second nicking enzyme that is capable of nicking upstream, downstream, or at the nicking site of said reverse template and does not nick within said target sequence; and
d) providing a DNA polymerase;

under essentially isothermal conditions, wherein amplification is performed by multiple cycles of said polymerase extending said forward and reverse templates along said target sequence producing a double-stranded nicking site, and said nicking enzymes nicking at said nicking sites, or amplified copies of said sites, producing an amplification product.
43. A method for amplifying a single-stranded nucleic acid target sequence, comprising

a) contacting a target nucleic acid comprising a single-stranded target sequence with a reverse template, wherein said reverse template comprises a nucleic acid sequence comprising a recognition region at the 3' end that is complementary to the 3' end of the target sequence, a nicking enzyme binding site and a nicking site upstream of said recognition region, and a stabilizing region upstream of said nicking site, wherein the portion of the nucleic acid sequence that is complementary to the 3' end of the target sequence is 8-15 nucleotides in length;
b) providing a first nicking enzyme that is capable of nicking at the nicking site of said reverse template, and does not nick within said target sequence;
c) providing a DNA polymerase under conditions wherein said polymerase extends said reverse template along said target sequence;
d) contacting said extended reverse template with a forward template, wherein said forward template comprises a recognition region at the 3'end that is complementary to the 3' end of the extended reverse template, a nicking enzyme binding site and a nicking site upstream of said recognition region, and a stabilizing region upstream of said nicking site, wherein the portion of the nucleic acid sequence that is complementary to the 3' end of the target antisense strand is 8-15 nucleotides in length;
e) providing a second nicking enzyme that is capable of nicking at the nicking site of said forward template and does not nick within said target sequence or within the complement of said target sequence;

wherein the amplification is conducted under essentially isothermal conditions wherein amplification is performed by multiple cycles of said polymerase extending said forward and reverse templates along said target sequence, producing double-stranded nicking sites, and said nicking enzymes nicking at said nicking sites, producing an amplification product.
44. The method of paragraph 42 or 43, wherein said DNA polymerase is a thermophilic polymerase.
45. The method of paragraph 42 or 43, wherein said polymerase is selected from the group consisting of Bst (large fragment), 9°N, $Vent_R$® (exo-) DNA Polymerase, Therminator, and Therminator II.
46. The method of paragraph 42 or 43, wherein said polymerase is Bst(large fragment).
47. The method of paragraph 42 or 43, wherein said nicking enzymes nick downstream of the nicking enzyme binding site.
48. The method of paragraph 42 or 43, wherein said forward and reverse templates comprise nicking enzyme binding sites recognized by the same nicking enzyme and said first and said second nicking enzymes are the same.
49. The method of paragraph 42 or 43, wherein said nicking enzymes are selected from the group consisting of Nt.BspQI, Nb.BbvCi, Nb.BsmI, Nb.BsrDI, Nb.BtsI, Nt.AlwI, Nt.BbvCI, Nt.BstNBI, Nt.CviPII, Nb.Bpu10I, and Nt.Bpu10I.

50. The method of paragraph 42 or 43, wherein said target sequence comprises from 1 to 5 nucleotides more than the sum of the nucleotides of said forward template recognition region and said reverse template recognition region.
51. The method of paragraph 42 or 43, wherein said target sequence comprises I nucleotide more than the sum of the nucleotides of said forward template recognition region and said reverse template recognition region.
52. The method of paragraph 42 or 43, wherein said target sequence comprises 2 nucleotides more than the sum of the nucleotides of said forward template recognition region and said reverse template recognition region.
53. The method of paragraph 42, wherein the target DNA molecule is selected from the group consisting of genomic DNA, plasmid, mitochondrial, and viral DNA.
54. The method of paragraph 43, wherein the target nucleic acid is selected from the group consisting of viral DNA, messenger RNA, microRNA, and microRNA precursors.
55. The method of paragraph 42 or 43, wherein the forward template is provided at the same concentration as the reverse template.
56. The method of paragraph 42 or 43, wherein one of the forward or reverse templates is provided at a ratio to the other template at the range of ratios of 1:100 to 100:1 1
57. The method of paragraph 43, further comprising a second polymerase.
58. The method of paragraph 57, wherein at least one of said polymerases comprises reverse transcriptase activity.
59. The method of paragraph 42 or 43, wherein the amplification is conducted between 54°C and 60°C.
60. The method of 42 or 43, wherein the amplification reaction is held at a constant temperature for 1 to 10 minutes.
61. The method of paragraph 42 or 43, further comprising detecting the amplification product.
62. The method of paragraph 42 or 43, wherein said amplification product is detected by a method selected from the group consisting of gel electrophoresis, mass spectrometry, SYBR I fluorescence, SYBR II fluorescence, SYBR Gold, Pico Green, TOTO-3, intercalating dye detection, FRET, molecular beacon detection, surface capture, capillary electrophoresis, incorporation of labeled nucleotides to allow detection by capture, fluorescence polarization, and lateral flow capture.
63. The method of paragraph 42 or 43, wherein at least two target sequences are capable of being amplified.
64. The method of paragraph 42 or 43, wherein said amplification products are detected on a solid surface.
65. The method of paragraph 42 or 43, wherein at least one capture probe is immobilized on a solid surface.
66. The method of paragraph 42 or 43, wherein at least one of said templates comprises a spacer, blocking group, or a modified nucleotide.
67. A method for amplifying a double-stranded nucleic acid target sequence, comprising

a) contacting a target DNA molecule comprising a double-stranded target sequence, having a sense strand and an antisense strand, with a forward template and a reverse template, wherein

i) said forward template comprises a nucleic acid sequence comprising a recognition region at the 3' end that is complementary to the 3' end of the target sequence antisense strand; a nicking enzyme binding site and a nicking site upstream of said recognition region and a stabilizing region upstream of said nicking site;
ii) said reverse template comprises a nucleotide sequence comprising a recognition region at the 3' end that is complementary to the 3' end of the target sequence sense strand, a nicking enzyme binding site and a nicking site upstream of said recognition region, and a stabilizing region upstream of said nicking site; and
iii) said target sequence comprises from 1 to 5 nucleotides more than the sum of the nucleotides of said forward template recognition region and said reverse template recognition region;

b) providing a first nicking enzyme that is capable of nicking at the nicking site of said forward template, and does not nick within said target sequence;
c) providing a second nicking enzyme that is capable of nicking at the nicking site of said reverse template and does not nick within said target sequence; and
d) providing a DNA polymerase;

under essentially isothermal conditions, wherein amplification is performed by multiple cycles of said polymerase extending said forward and reverse templates along said target sequence producing a double-stranded nicking site, and said nicking enzymes nicking at said nicking sites, or amplified copies of said sites, producing an amplification product.
68. A method for amplifying a single-stranded nucleic acid target sequence, comprising

a) contacting a target nucleic acid comprising a single-stranded target sequence with a reverse template, wherein said reverse template comprises a nucleic acid sequence comprising a recognition region at the 3' end that is

complementary to the 3' end of the target sequence, a nicking enzyme binding site and a nicking site upstream of said recognition region, and a stabilizing region upstream of said nicking site, wherein the portion of the nucleic acid sequence that is complementary to the 3' end of the target sequence is 8-15 nucleotides in length;
b) providing a first nicking enzyme that is capable of nicking at the nicking site of said reverse template, and does not nick within said target sequence;
c) providing a DNA polymerase under conditions wherein said polymerase extends said reverse template along said target sequence;
d) contacting said extended reverse template with a forward template, wherein said forward template comprises a recognition region at the 3'end that is complementary to the 3' end of the extended reverse template, a nicking enzyme binding site and a nicking site upstream of said recognition region, and a stabilizing region upstream of said nicking site, wherein said target sequence comprises from 1 to 5 nucleotides more than the sum of the nucleotides of said forward template recognition region and said reverse template recognition region;
e) providing a second nicking enzyme that is capable of nicking at the nicking site of said forward template and does not nick within said target sequence or within the complement of said target sequence;

wherein the amplification is conducted under essentially isothermal conditions wherein amplification is performed by multiple cycles of said polymerase extending said forward and reverse templates along said target sequence, producing double-stranded nicking sites, and said nicking enzymes nicking at said nicking sites, producing an amplification product.
69. The method of paragraph 67 or 68, wherein said target sequence comprises I nucleotide more than the sum of the nucleotides of said forward template recognition region and said reverse template recognition region.
70. The method of paragraph 67 or 68, wherein said target sequence comprises 2 nucleotides more than the sum of the nucleotides of said forward template recognition region and said reverse template recognition region.
71. The method of paragraph 67 or 68, wherein said target sequence comprises 3 nucleotides more than the sum of the nucleotides of said forward template recognition region and said reverse template recognition region.
72. A method for amplifying a double-stranded nucleic acid target sequence, comprising

a) contacting a target DNA molecule comprising a double-stranded target sequence, having a sense strand and an antisense strand, with a forward template and a reverse template, wherein
b) said forward template comprises a nucleic acid sequence comprising a recognition region at the 3' end that is complementary to the 3' end of the target sequence antisense strand; a nicking enzyme binding site and a nicking site upstream of said recognition region and a stabilizing region upstream of said nicking site;
c) said reverse template comprises a nucleotide sequence comprising a recognition region at the 3' end that is complementary to the 3' end of the target sequence sense strand, a nicking enzyme binding site and a nicking site upstream of said recognition region, and a stabilizing region upstream of said nicking site;
d) providing a first nicking enzyme that is capable of nicking at the nicking site of said forward template, and does not nick within said target sequence;
e) providing a second nicking enzyme that is capable of nicking at the nicking site of said reverse template and does not nick within said target sequence; and
f) providing a DNA polymerase;

under essentially isothermal conditions, wherein amplification is performed by multiple cycles of said polymerase extending said forward and reverse templates along said target sequence producing a double-stranded nicking site, and said nicking enzymes nicking at said nicking sites, or amplified copies of said sites, producing an amplification product.
73. A method for amplifying a double-stranded nucleic acid target sequence, comprising

a) contacting a target DNA molecule comprising a double-stranded target sequence, having a sense strand and an antisense strand, with a forward template and a reverse template, wherein
b) said forward template comprises a nucleic acid sequence comprising a recognition region at the 3' end that is complementary to the 3' end of the target sequence antisense strand; a nicking enzyme binding site and a nicking site upstream of said recognition region and a stabilizing region upstream of said nicking site;

ii) said reverse template comprises a nucleotide sequence comprising recognition region at the 3' end that is complementary to the 3' end of the target sequence sense strand, a nicking enzyme binding site and a nicking site upstream of said recognition region, and a stabilizing region upstream of said nicking site;

c) providing a first nicking enzyme that is capable of nicking upstream, downstream, or at the nicking site of

said forward template, and does not nick within said target sequence;
d) providing a second nicking enzyme that is capable of nicking upstream, downstream, or at the nicking site of said reverse template and does not nick within said target sequence; and
e) providing a DNA polymerase;

under essentially isothermal conditions, wherein amplification is performed by multiple cycles of said polymerase extending said forward and reverse templates along said target sequence producing a double-stranded nicking site, and said nicking enzymes nicking at said nicking sites, or amplified copies of said sites, producing an amplification product.

74. A method for amplifying a double-stranded nucleic acid target sequence, comprising

a) contacting a target DNA molecule comprising a double-stranded target sequence, having a sense strand and an antisense strand, with a forward template and a reverse template, wherein
b) said forward template comprises a nucleic acid sequence comprising a recognition region at the 3' end that is complementary to the 3' end of the target sequence antisense strand; a nicking enzyme binding site and a nicking site upstream of said recognition region and a stabilizing region upstream of said nicking site;
c) said reverse template comprises a nucleotide sequence comprising a recognition region at the 3' end that is complementary to the 3' end of the target sequence sense strand, a nicking enzyme binding site and a nicking site upstream of said recognition region, and a stabilizing region upstream of said nicking site;
d) providing a first nicking enzyme that is capable of nicking at the nicking site of said forward template, and does not nick within said target sequence;
e) providing a second nicking enzyme that is capable of nicking at the nicking site of said reverse template and does not nick within said target sequence; and
f) providing a DNA polymerase;

under essentially isothermal conditions, wherein amplification is performed by multiple cycles of said polymerase extending said forward and reverse templates along said target sequence producing a double-stranded nicking site, and said nicking enzymes nicking at said nicking sites, or amplified copies of said sites, producing an amplification product, wherein at least a 1E + 7 fold amplification of a 22-35 nucleotide long target sequence is obtained when the amplification reaction is run for twelve minutes.

75. A method for amplifying a double-stranded nucleic acid target sequence, comprising

a) contacting a target DNA molecule comprising a double-stranded target sequence, having a sense strand and an antisense strand, with a forward template and a reverse template, wherein
b) said forward template comprises a nucleic acid sequence comprising a recognition region at the 3' end that is complementary to the 3' end of the target sequence antisense strand; a nicking enzyme binding site and a nicking site upstream of said recognition region and a stabilizing region upstream of said nicking site, wherein the portion of the nucleic acid sequence that is complementary to the 3' end of the target antisense strand is 8-15 nucleotides in length;
c) said reverse template comprises a nucleotide sequence comprising recognition region at the 3' end that is complementary to the 3' end of the target sequence sense strand, a nicking enzyme binding site and a nicking site upstream of said recognition region, and a stabilizing region upstream of said nicking site, wherein the portion of the nucleic acid sequence that is complementary to the 3' end of the target antisense strand is 8-15 nucleotides in length;
d) providing a first nicking enzyme that is capable of nicking upstream, downstream, or at the nicking site of said forward template, and does not nick within said target sequence;
e) providing a second nicking enzyme that is capable of nicking upstream, downstream, or at the nicking site of said reverse template and does not nick within said target sequence; and
f) providing a DNA polymerase;

under essentially isothermal conditions, wherein amplification is performed by multiple cycles of said polymerase extending said forward and reverse templates along said target sequence producing a double-stranded nicking site, and said nicking enzymes nicking at said nicking sites, or amplified copies of said sites, producing an amplification product, wherein at least a 1E + 7 fold amplification of a 22-35 nucleotide long target sequence is obtained when the amplification reaction is run for twelve minutes.

76. A kit for amplifying a nucleic acid target sequence, comprising

a) a DNA polymerase;

b) a first template for nucleic acid amplification, comprising a recognition region at the 3' end that is complementary to the 3' end of a target sequence sense strand; a nicking enzyme binding site and a nicking site upstream of said recognition region; and a stabilizing region upstream of said nicking site, wherein the portion of the nucleic acid sequence that is complementary to the 3' end of the target sequence sense strand is 8-15 nucleotides in length;
c) a second template for nucleic acid amplification, comprising a recognition region at the 3' end that is complementary to the 3' end of the complement of the target sequence sense strand; a nicking enzyme binding site and a nicking site upstream of said recognition region; and a stabilizing region upstream of said nicking site, wherein the portion of the nucleic acid sequence that is complementary to the 3' end of the complement of the target sequence sense strand is 8-15 nucleotides in length; and
d) one or two thermostable nicking enzymes, wherein either one enzyme is capable of nicking at the nicking site of said first and said second templates, or a first enzyme is capable of nicking at the nicking site of said first primer and a second enzyme is capable of nicking at the enzyme site of said second primer.

77. The kit of paragraph 76, wherein said target sequence comprises from 1 to 5 nucleotides more than the sum of the nucleotides of said first template recognition region and said second template recognition region.
78. The kit of paragraph 76, wherein said polymerase, nicking enzymes, and templates are in a container.
79. The kit of paragraph 76, wherein said polymerase, nicking enzymes, and templates are in two containers.
80. The kit of paragraph 76, wherein said polymerase and nicking enzymes are in a first container, and said templates are in a second container.
81. The kit of paragraph 76, wherein said polymerase, nicking enzymes, and templates are lyophilized.
82. The kit of paragraph 76, further comprising instructions for following the method of amplification.
83. The kit of paragraph 76, further comprising a cuvette.
84. The kit of paragraph 76 further comprising a lateral flow device or dipstick.
85. The kit of paragraph 84, wherein said lateral flow device or dipstick further comprises a capture probe.
86. The kit of paragraph 76, further comprising a detector component selected from the group consisting of a fluorescent dye, colloidal gold particles, latex particles, a molecular beacon, and polystyrene beads.
87. The kit of paragraph 76, wherein at least one of said templates comprises a spacer, blocking group, or a modified nucleotide.
88. A kit for amplifying a nucleic acid target sequence, comprising

a) a DNA polymerase;
b) a first template for nucleic acid amplification, comprising a recognition region at the 3' end that is complementary to the 3' end of a target sequence sense strand; a nicking enzyme binding site and a nicking site upstream of said recognition region; and a stabilizing region upstream of said nicking site;
c) a second template for nucleic acid amplification, comprising a recognition region at the 3' end that is complementary to the 3' end of the complement of the target sequence sense strand; a nicking enzyme binding site and a nicking site upstream of said recognition region; and a stabilizing region upstream of said nicking site, wherein said target sequence comprises from 1 to 5 nucleotides more than the sum of the nucleotides of said first template recognition region and said second template recognition region; and
d) one or two thermostable nicking enzymes, wherein either one enzyme is capable of nicking at the nicking site of said first and said second templates, or a first enzyme is capable of nicking at the nicking site of said first primer and a second enzyme is capable of nicking at the enzyme site of said second primer.

89. The kit of paragraph 88, wherein the portion of the nucleic acid sequence of the first template that is complementary to the 3' end of the target sequence sense strand is 8-15 nucleotides in length, and the portion of the nucleic acid sequence of the second template that is complementary to the 3' end of the complement of the target sequence sense strand is 8-15 nucleotides in length.
90. The kit of paragraph 88, wherein said polymerase, nicking enzymes, and templates are in a container.
91. The kit of paragraph 88, wherein said polymerase, nicking enzymes, and templates are in two containers.
92. The kit of paragraph 88, wherein said polymerase and nicking enzymes are in a first container, and said templates are in a second container.
93. The kit of paragraph 88, wherein said polymerase, nicking enzymes, and templates are lyophilized.
94. The kit of paragraph 88, further comprising instructions for following the method of amplification.
95. The kit of paragraph 88, further comprising a cuvette.
96. The kit of paragraph 88 further comprising a lateral flow device or dipstick.
97. The kit of paragraph 96, wherein said lateral flow device or dipstick further comprise a capture probe.
98. The kit of paragraph 88, further comprising a detector component selected from the group consisting of a

fluorescent dye, colloidal gold particles, latex particles, a molecular beacon, and polystyrene beads.
99. The kit of paragraph 88, wherein at least one of said templates comprises a spacer, blocking group, or a modified nucleotide.

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SEQUENCE LISTING

<110> IONIAN TECHNOLOGIES, INC.

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<120> NICKING AND EXTENSION AMPLIFICATION REACTION
FOR THE EXPONENTIAL AMPLIFICATION OF NUCLEIC
ACIDS

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<151> 2007-07-14

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20

Claims

1. A method for nucleotide sequence amplification, which comprises:

25

combining a target nucleic acid having a target nucleotide sequence with (i) a polymerase, (ii) a first template nucleic acid that hybridizes to a first strand of the target nucleotide sequence, and (iii) a second template nucleic acid that hybridizes to the complement of the first strand of the target nucleotide sequence, in an amplification reaction, under conditions in which the polymerase extends the template nucleic acids, thereby generating extended template nucleic acid amplicons; wherein:

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the target nucleotide sequence is between 20 and 40 nucleotides in length;
the target nucleotide sequence is amplified 1E+6-fold or more in about ten minutes;
said method uses a nicking enzyme; and

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the foregoing steps are conducted under substantially isothermal conditions.

2. The method of claim 1, wherein the first template comprises a nucleic acid sequence comprising a first template recognition region at the 3' end that is complementary or substantially complementary to a region of the first strand of the target nucleotide sequence; and the second template comprises a nucleotide sequence comprising a second

40

template recognition region at the 3' end that is complementary or substantially complementary to a region of the complement of the first strand of the target nucleotide sequence, optionally wherein said first template recognition region is complementary or substantially complementary to the 3' end of the first strand of the target nucleotide sequence and said second template recognition region is complementary or substantially complementary to the 3' end of the complement of the first strand of the target nucleotide sequence.

45

3. The method of claim 2, wherein the first template and second template comprise nicking enzyme binding sites and nicking sites upstream of the recognition regions, and the amplification reaction further comprises one or more nicking enzymes that are capable of nicking at the nicking site of said first and said second templates, wherein either one nicking enzyme is capable of nicking both of said templates, or each template is capable of being nicked by at

50

least one of the nicking enzymes, and wherein said one or more nicking enzymes do not nick within said target sequence.

4. The method of claim 3, wherein:

55

(i) the first and second templates comprise nicking enzyme binding sites recognized by the same nicking enzyme and said first and said second nicking enzyme are the same; and/or
(ii) said nicking enzyme is selected from the group of nicking enzymes shown in Table 3 or from the group of nicking enzymes consisting of Nt.BspQI, Nb.BbvCi, Nb.BsmI, Nb.BsrDI, Nb.BtsI, NtAlwI, NtBbvCI, NtBstNBI,

Nt.CviPII, Nb.BpuIOI, and Nt.BpuIOI, preferably wherein the nicking enzyme is selected from the group consisting of Nt.Bst.NBI, Nb.BsmI and Nb.BsrDI.

5. The method of claim 3 or 4, wherein the target nucleotide sequence comprises 1, 2 or 3 nucleotides more than the sum of the nucleotides of the first template recognition region and the second template recognition region.

6. The method of any of claims 1-5, wherein the target nucleic acid is double stranded or single stranded, optionally wherein the target nucleic acid is:

(i) double-stranded DNA, e.g. wherein the target nucleic acid is selected from the group consisting of genomic DNA, plasmid DNA, viral DNA, mitochondrial DNA, and synthetic double-stranded DNA;
(ii) single-stranded DNA, e.g. wherein the target nucleic acid is selected from the group consisting of viral DNA, cDNA, and synthetic single-stranded DNA;
(iii) RNA, e.g. wherein the target nucleic acid is selected from the group consisting of messenger RNA, viral RNA, ribosomal RNA, transfer RNA, micro RNA, micro RNA precursor, and synthetic RNA.

7. The method of any of claims 1-6, wherein either:

(i) said DNA polymerase is a thermophilic polymerase;
(ii) said polymerase is selected from the group consisting of Bst (large fragment), 9°N, $Vent_R$® (exo-) DNA Polymerase, Therminator, and Therminator II;
(iii) said polymerase is Bst (large fragment); or
(iv) said polymerase has strand-displacement capabilities.

8. The method of any of claims 1-7, wherein either:

(i) the portion of the nucleic acid sequence of the first template that is complementary or substantially complementary to the first strand of the target nucleotide sequence is 8-12, 8-13 or 8-15 nucleotides in length and wherein the portion of the second template that is complementary or substantially complementary to the complement of the first strand of the target nucleotide sequence is 8-12, 8-13 or 8-15 nucleotides in length; and/or
(ii) there is an internal hairpin within said template sequence.

9. The method of any of claims 1-8, wherein either:

(i) the first template is provided at the same concentration as the second template; or
(ii) one of the first or second templates is provided at a ratio to the other template at the range of ratios of 1 : 100 to 100: 1

10. The method of any of claims 1-9, further comprising a second polymerase, optionally wherein at least one of the first or second polymerases comprises reverse transcriptase activity.

11. The method of any of claims 1-10, wherein the amplification is conducted between:

(i) 54°C and 60°C; or
(ii) 56°C and 58°C

12. The method of any of claims 1-11, wherein either:

(i) the amplification reaction is held at a constant temperature for 1 to 10 or for 1 to 20 minutes; and/or
(ii) the temperature of the amplification reaction is higher than the initial melting temperature of the template/ target sequence complex.

13. The method of any of claims 1-12, further comprising detecting the amplification product, optionally wherein said amplification product is detected by a detection method selected from the group consisting of gel electrophoresis, mass spectrometry, SYBR I fluorescence, SYBR II fluorescence, SYBR Gold, Pico Green, TOTO-3, intercalating dye detection, fluorescence resonance energy transfer (FRET), molecular beacon detection, surface capture, capillary electrophoresis, incorporation of labeled nucleotides to allow detection by capture, fluorescence polarization, and lateral flow capture.

14. The method of any of claims 1-13, wherein at least two target sequences are capable of being amplified.

15. The method of any of claims 1-14, wherein:

5 (i) said amplification products are detected on a solid surface;
(ii) at least one capture probe is immobilized on a solid surface; and/or
(iii) at least one of said templates comprises a spacer, blocking group, or a modified nucleotide.

16. The method of any of claims 1-15, wherein the target nucleotide sequence is amplified:

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(i) 1E+6-fold or more in about 2.5 or 5 minutes; or
(ii) 1E+7-fold, 1E+8-fold 1E+9-fold or more in about five minutes.

17. The method of any of claims 1-16, wherein:

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i) said first template comprises a nucleic acid sequence comprising a recognition region at the 3' end that is complementary or substantially complementary to the 3' end of the first strand of the target nucleotide sequence, a nicking enzyme binding site and a nicking site upstream of said recognition region and a stabilizing region upstream of said nicking site; and
20 ii) said second template comprises a nucleotide sequence comprising a recognition region at the 3' end that is complementary or substantially complementary to the 3' end of the complement of the first strand of the target nucleotide sequence, a nicking enzyme binding site and a nicking site upstream of said recognition region, and a stabilizing region upstream of said nicking site; optionally wherein said stabilizing region has a GC content of at least 40% and/or a length of at least 6 nucleotides.

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18. A method for nucleotide sequence amplification, which comprises:

a) combining a target nucleic acid having a target nucleotide sequence with (i) a polymerase, (ii) a nicking enzyme and (iii) first and second primers;
30 b) performing an initial heat denaturation step; and
c) performing nicking and extension reactions to amplify the target nucleotide sequence under isothermal conditons;
wherein the nicking enzyme is selected from the group consisting of Nt.BspQI, Nb.BbvCI, Nb.BsmI, Nb.BsrDI, Nb.BtsI, Nt.AlwI, Nt.BbvCI and Nt.CviPII.

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19. A kit for amplifying a target nucleotide sequence, comprising

a) a DNA polymerase;
b) a first template nucleic acid that hybridizes to a first strand of the target nucleotide sequence;
40 c) a second template nucleic acid that hybridizes to the complement of the first strand of the target nucleotide sequence
d) a nicking enzyme.

20. The kit of claim 19, wherein said DNA polymerase, first and second template nucleic acids and nicking enzyme are
45 as defined in any preceding claim.

21. The kit of claim 19 or 20, wherein:

(i) said polymerase and nicking enzymes are in a first container, and said templates are in a second container;
50 (ii) said kit further comprises instructions for following the method of amplification;
(iii) said kit further comprises a detector component selected from the group consisting of a fluorescent dye, colloidal gold particles, latex particles, a molecular beacon, and polystyrene beads;
(iv) at least one of said templates comprises a spacer, blocking group, or a modified nucleotide; and/or
(v) said templates have a stabilizing region that has a GC content of at least 40% and/or a length of at least 6
55 nucleotides.

22. Use of gel electrophoresis, mass spectrometry, SYBR I fluorescence, SYBR II fluorescence, SYBR Gold, Pico Green, TOTO-3, intercalating dye detection, FRET, molecular beacon detection, surface capture, capillary electro-

phoresis, capture detection with labeled nucleotides, fluorescence polarization, lateral flow capture or a capture probe immobilized on a solid surface to detect a target sequence that has been amplified by a method of any one of claims 1-18.

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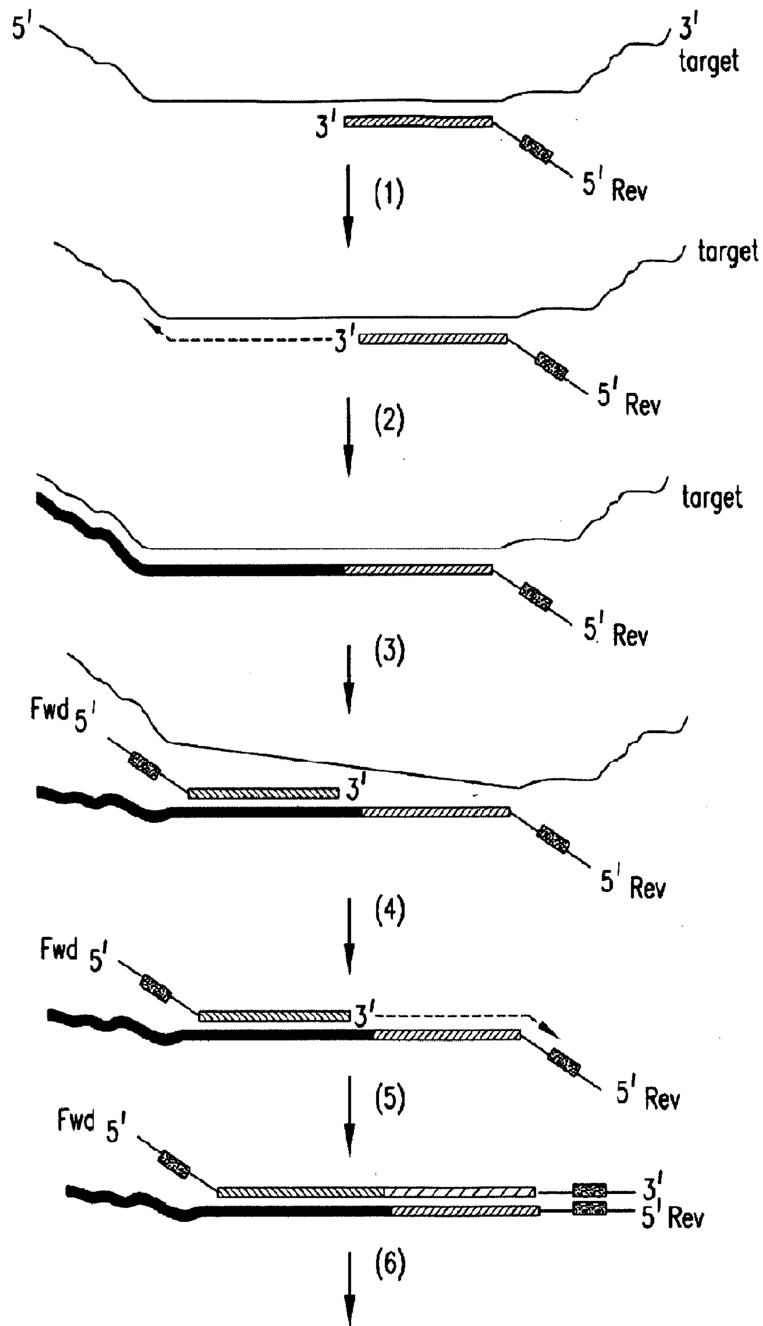

FIG.1A

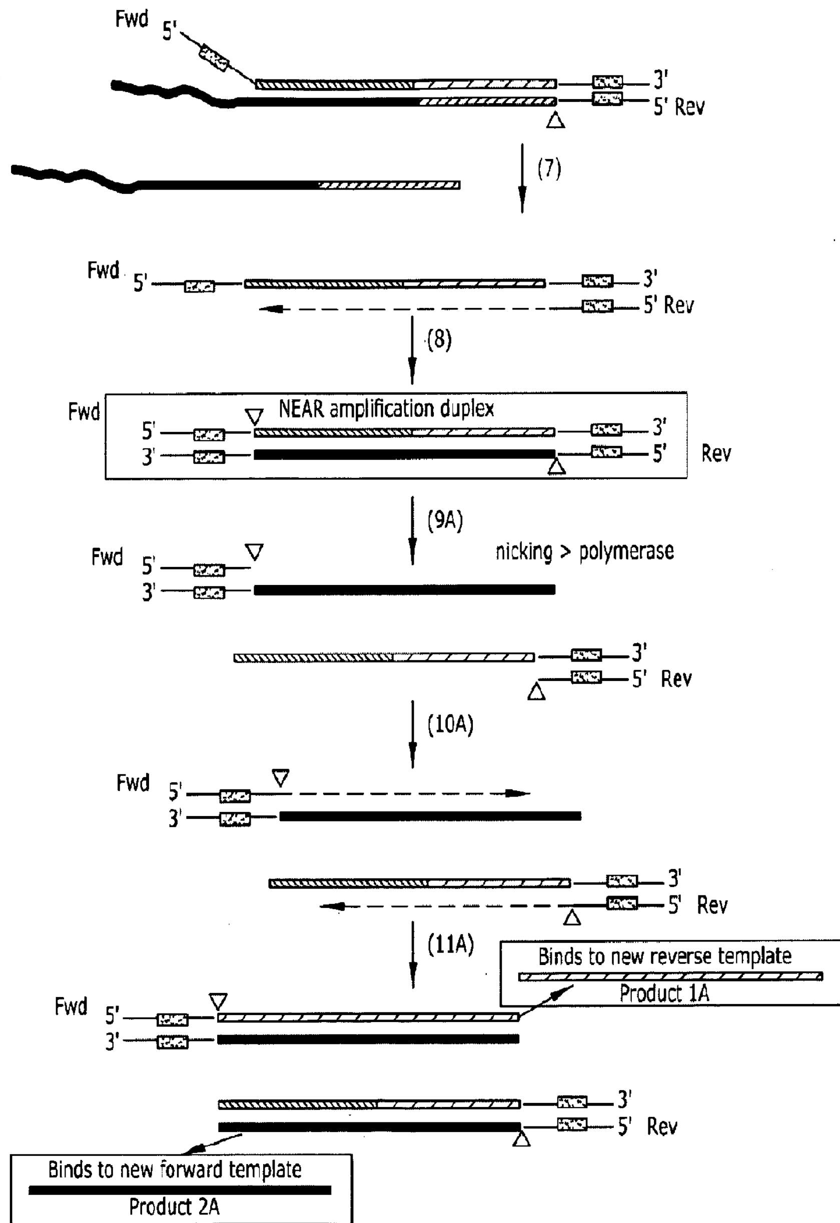

FIG. 1B

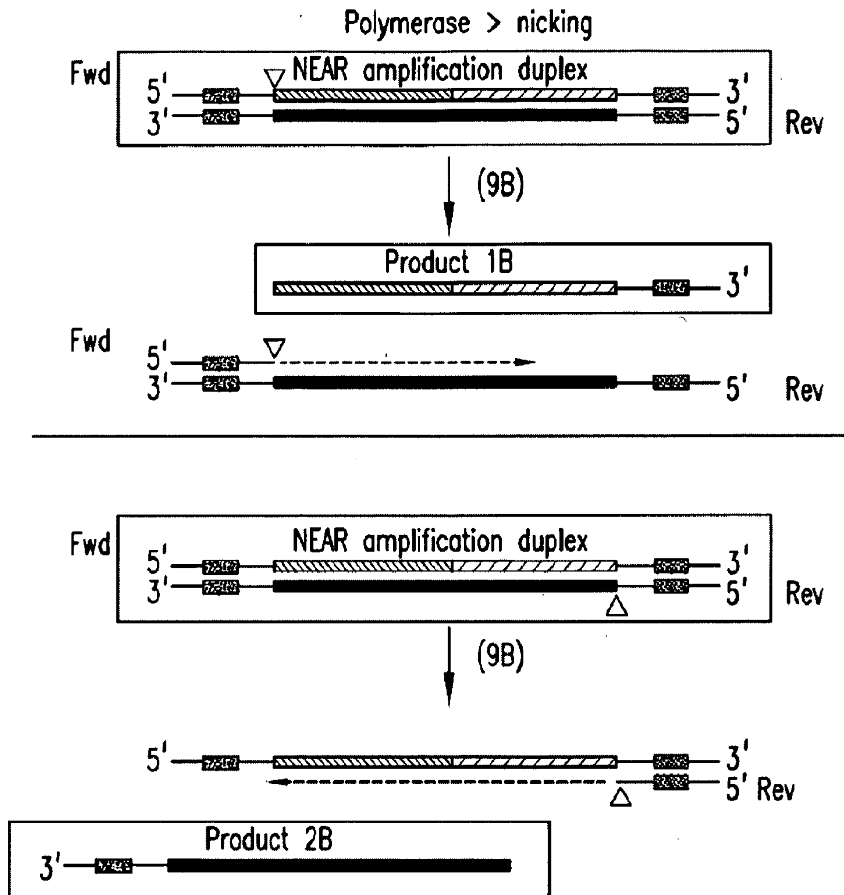

FIG.1C

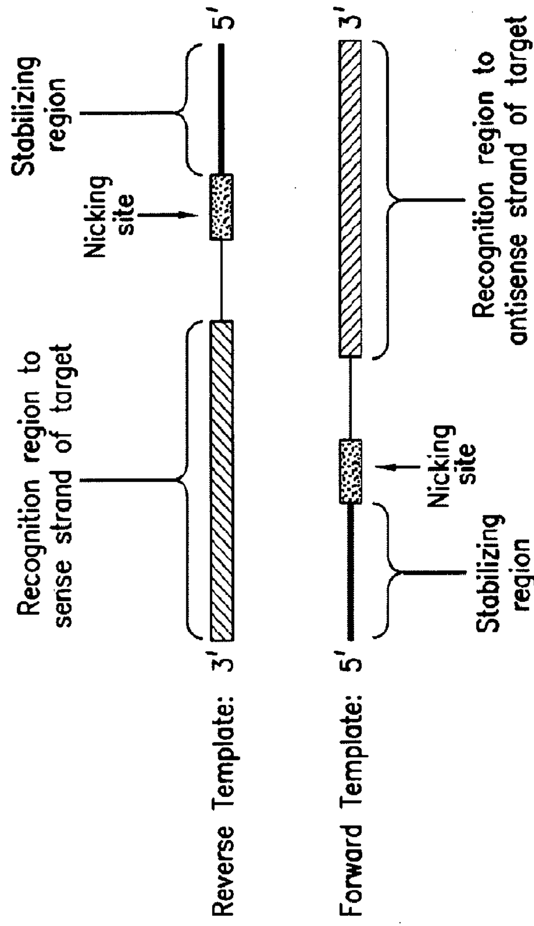

FIG.1D

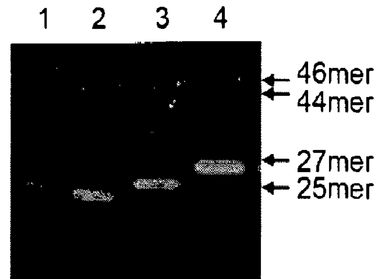

FIG.2

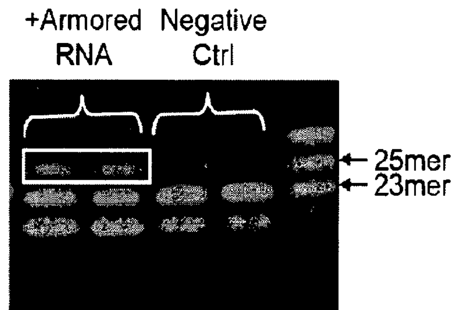

FIG.3

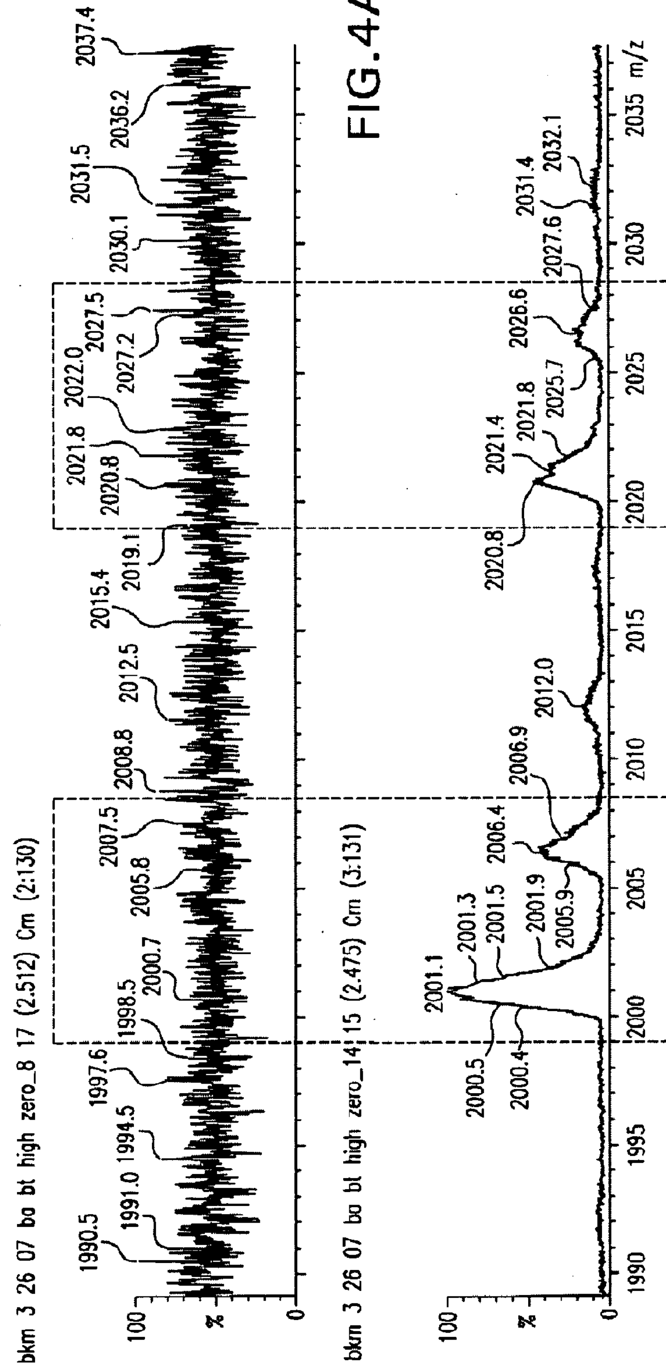

FIG.4A

FIG.4B

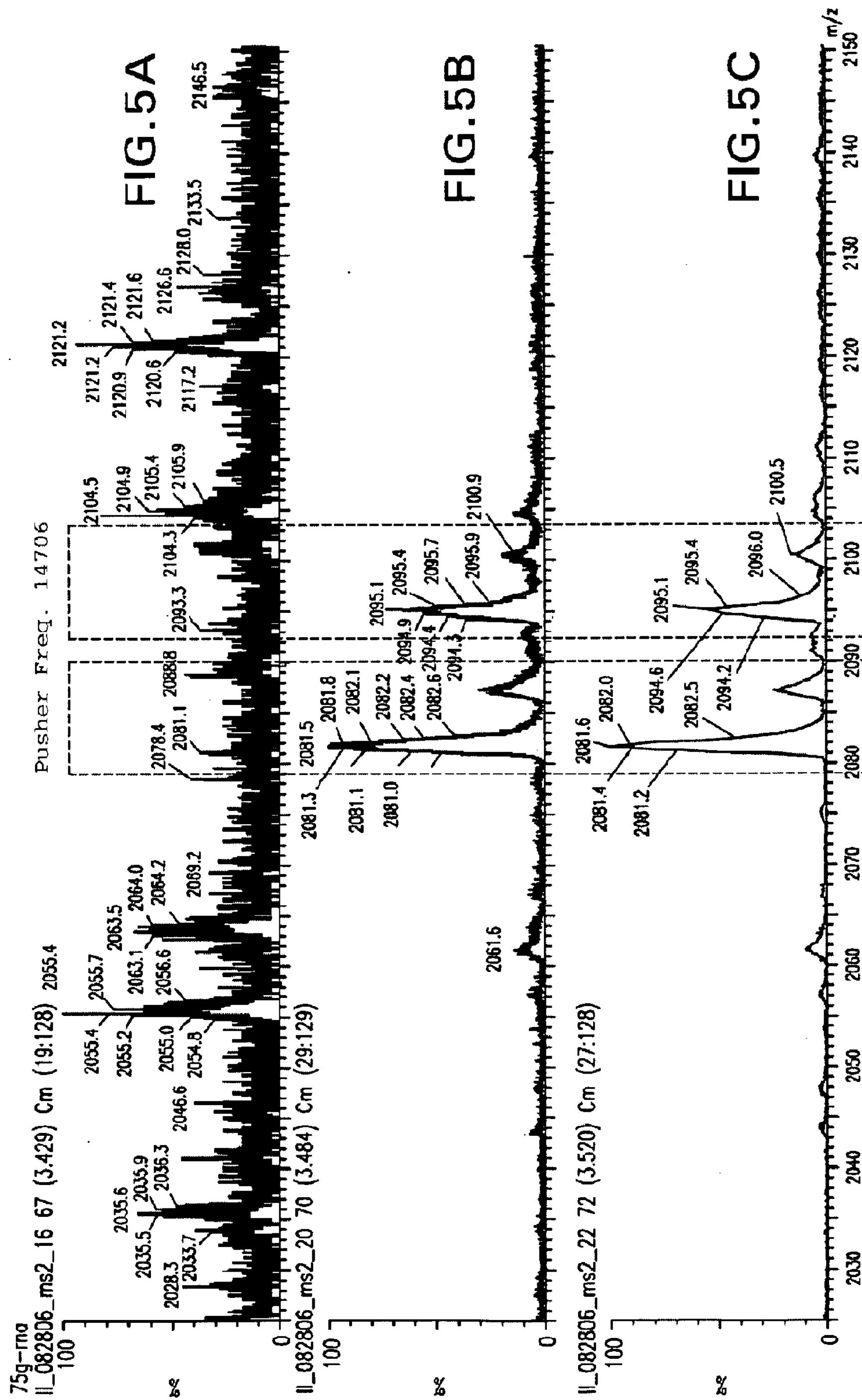

FIG.5A
FIG.5B
FIG.5C
Pusher Freq. 14706
75g-rna
II_082806_ms2_16 67 (3.429) Cm (19:128)
II_082806_ms2_20 70 (3.484) Cm (29:129)
II_082806_ms2_22 72 (3.520) Cm (27:128)
100
%
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m/z
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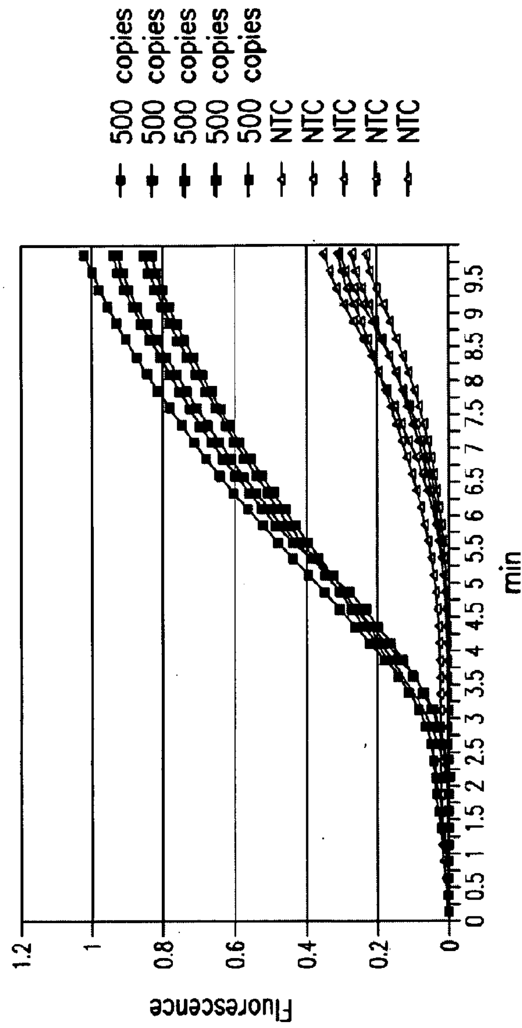
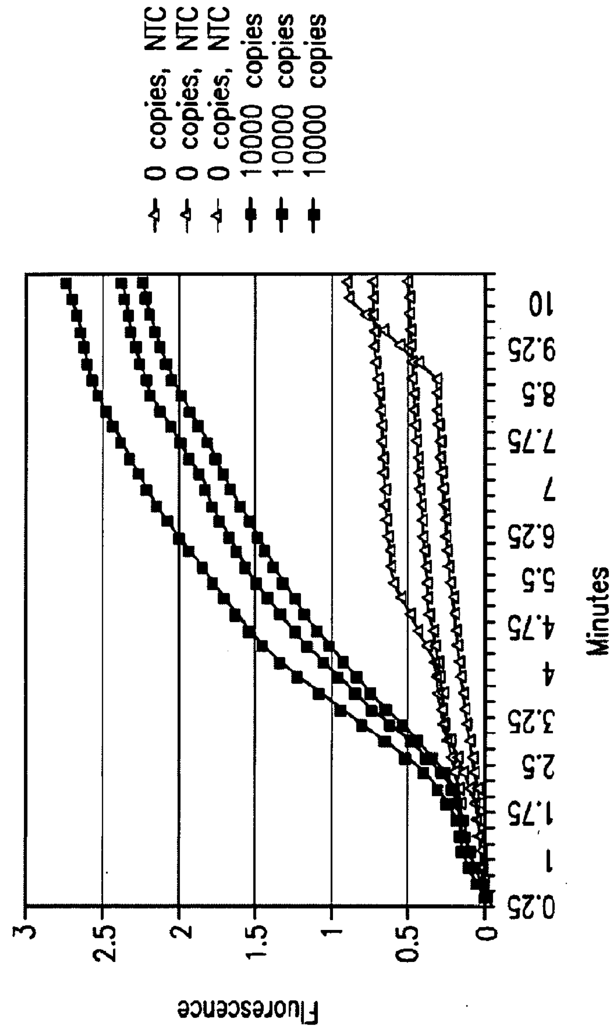


FIG.6



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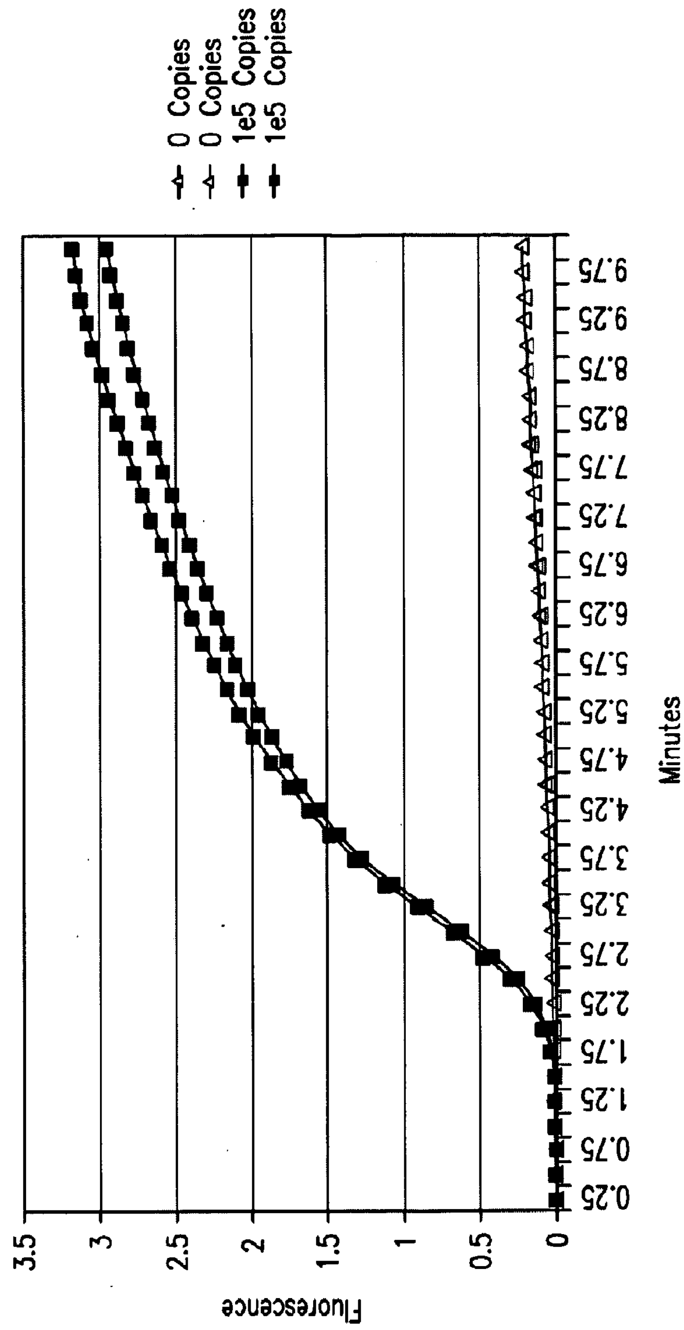


FIG.8

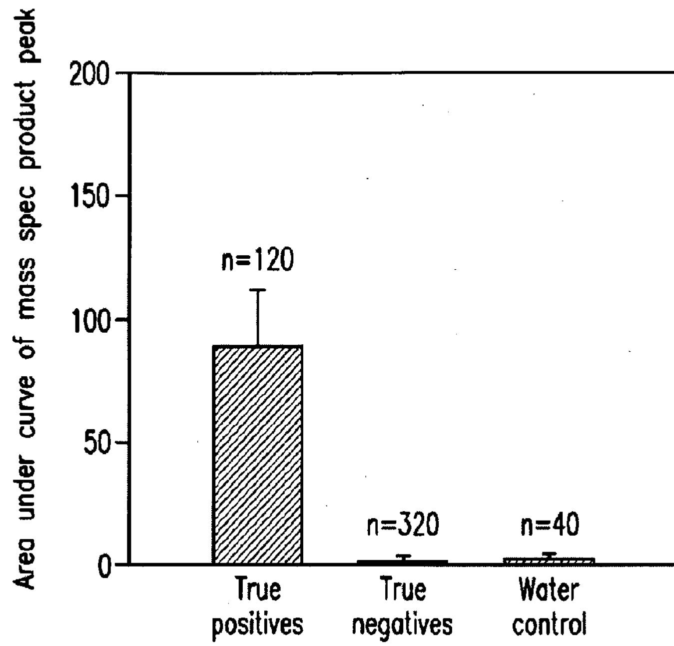


FIG.9

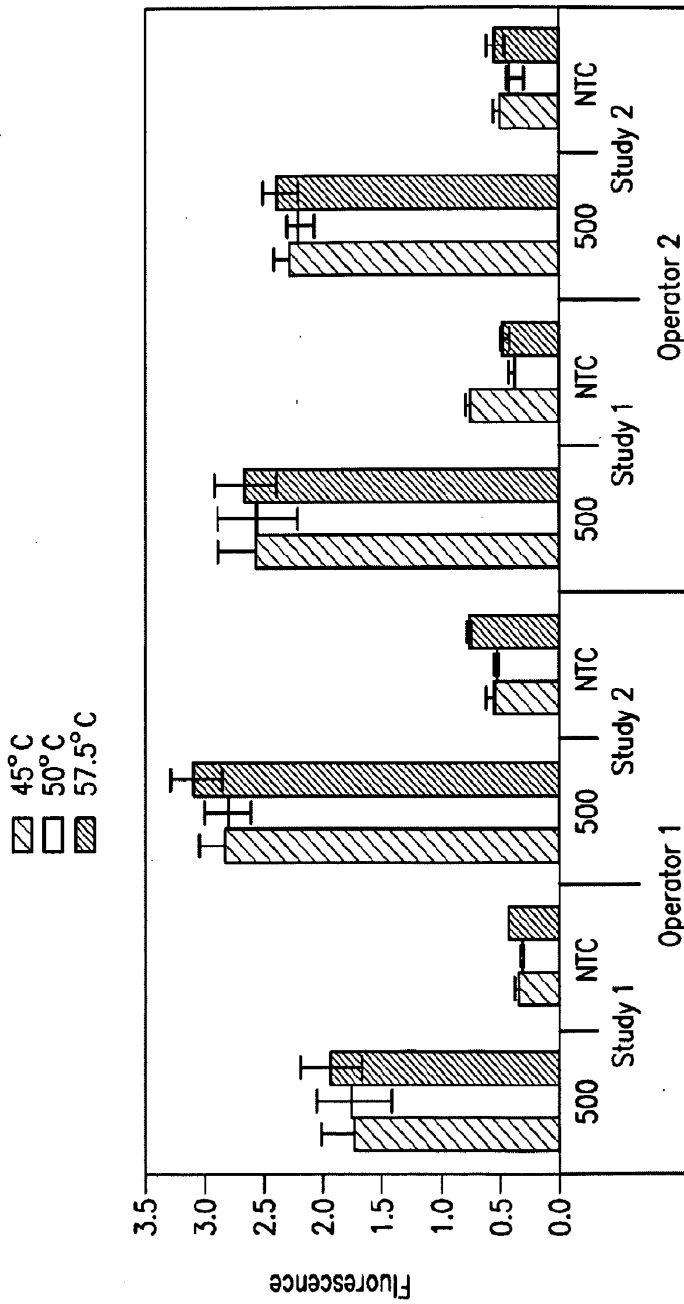


FIG.10

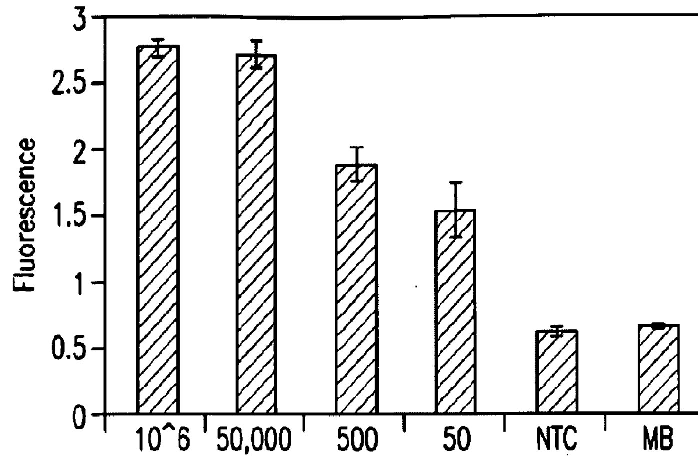


FIG.11

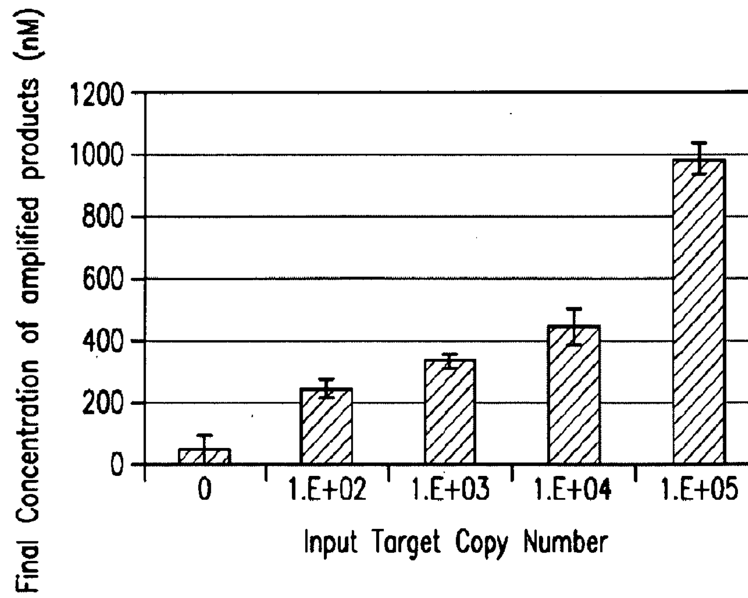


FIG.12

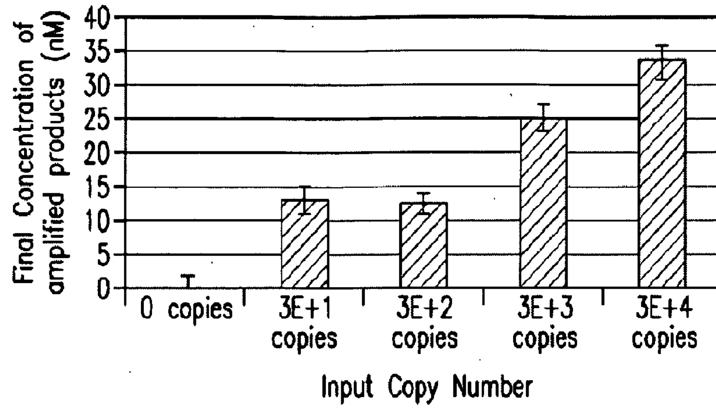


FIG.13

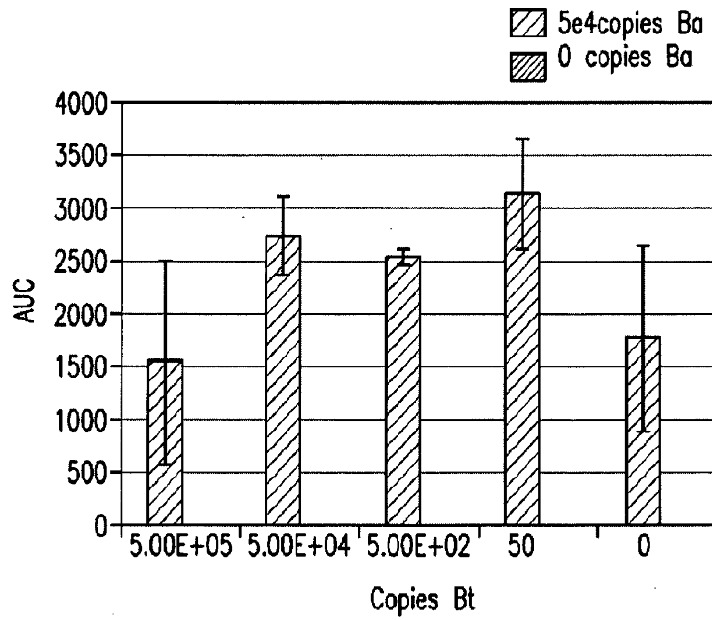


FIG.14

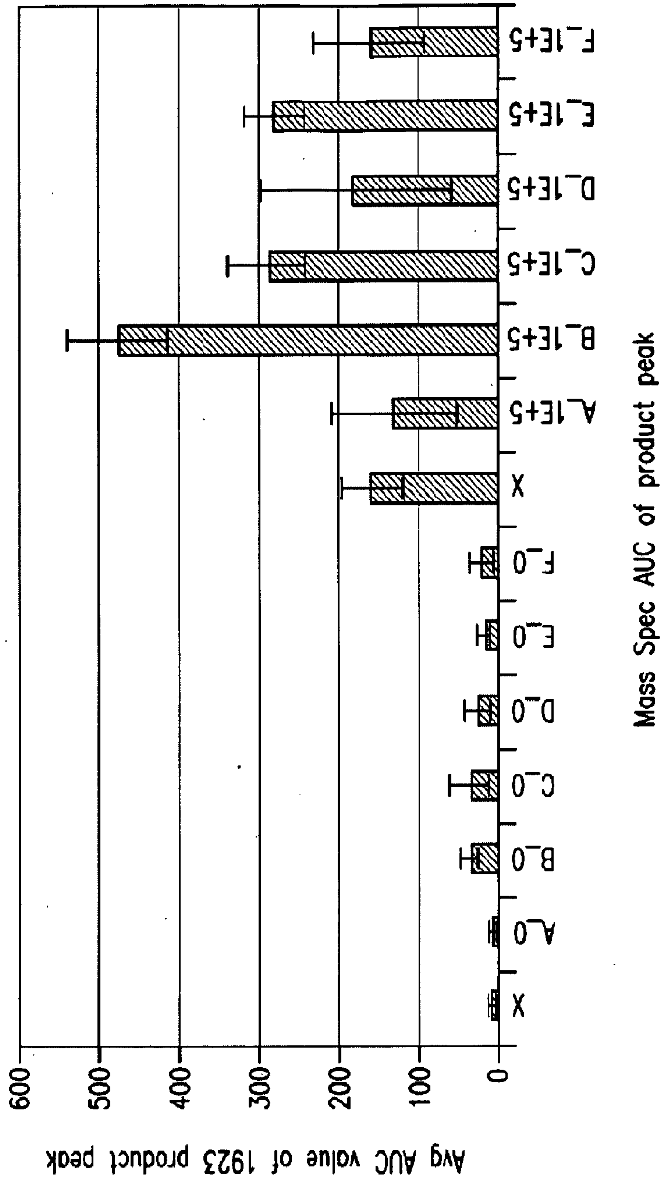


FIG.15

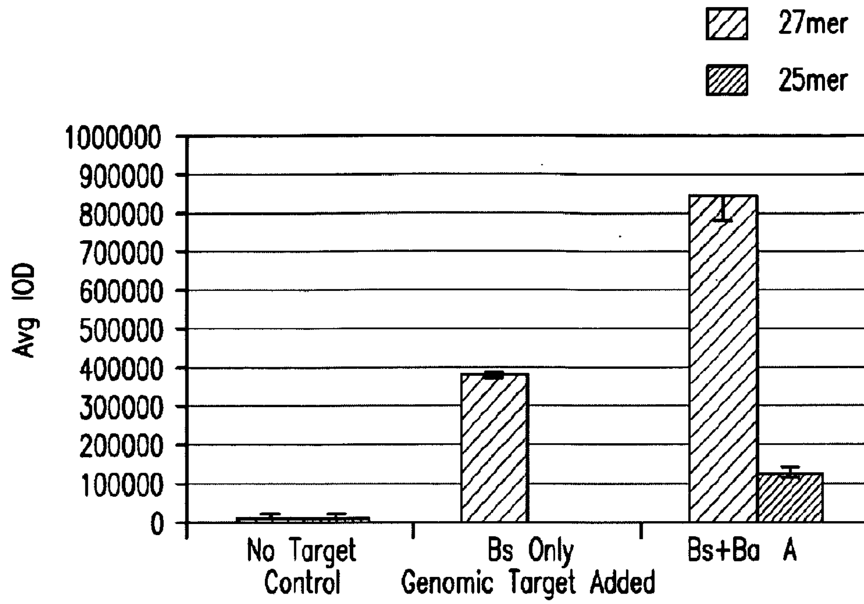


FIG.16

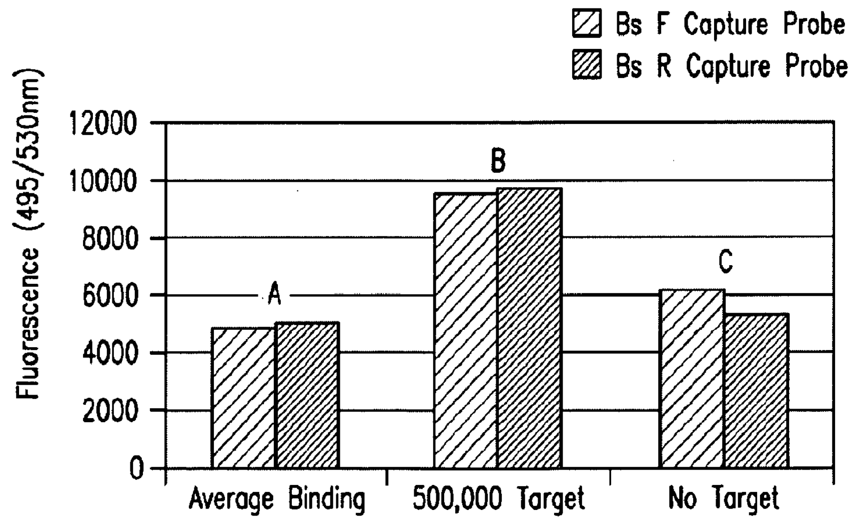


FIG.20

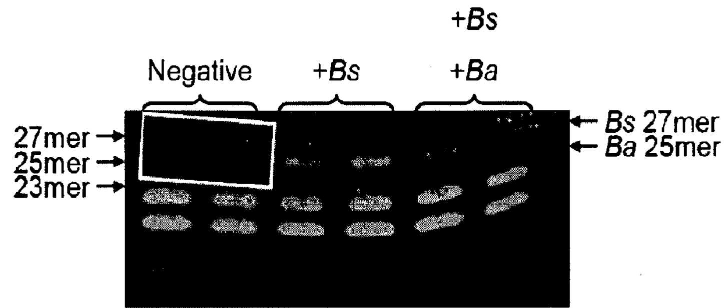


FIG.17

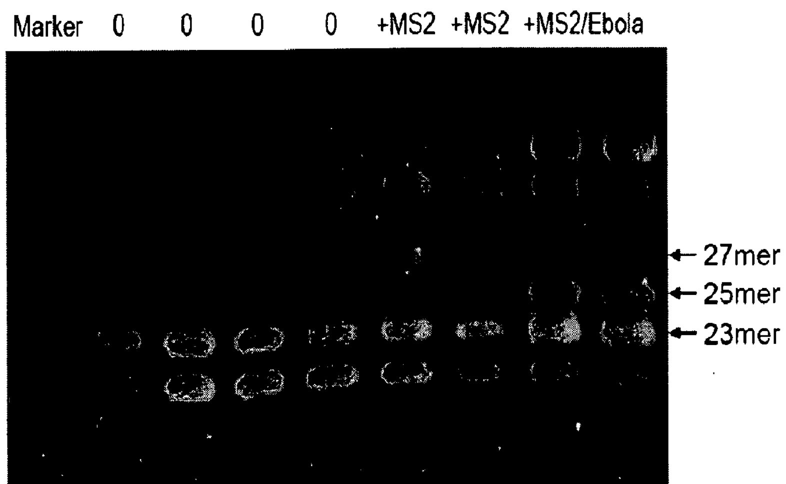


FIG.18

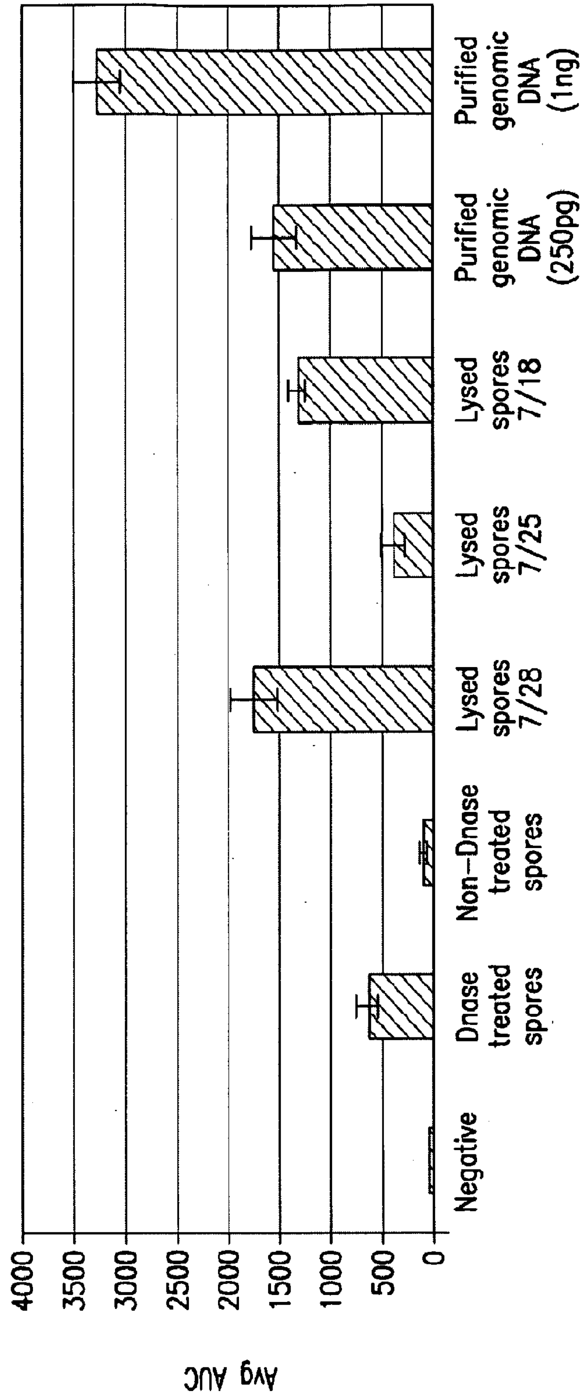


FIG.19

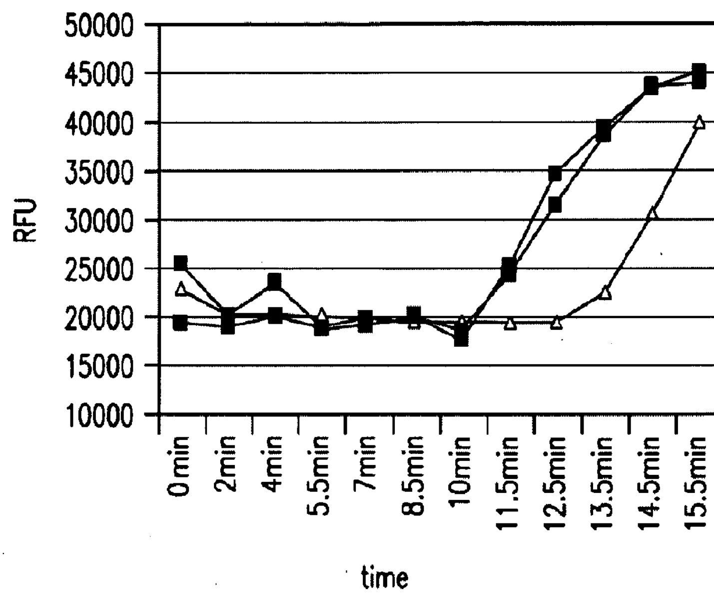


FIG.21

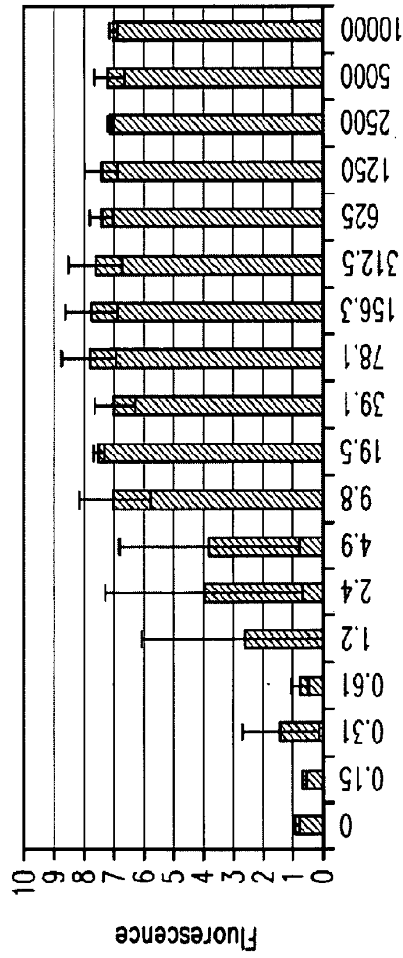


FIG.22A

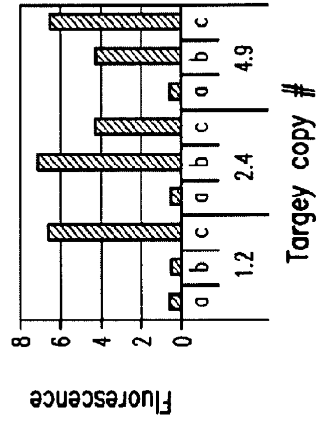


FIG.22B

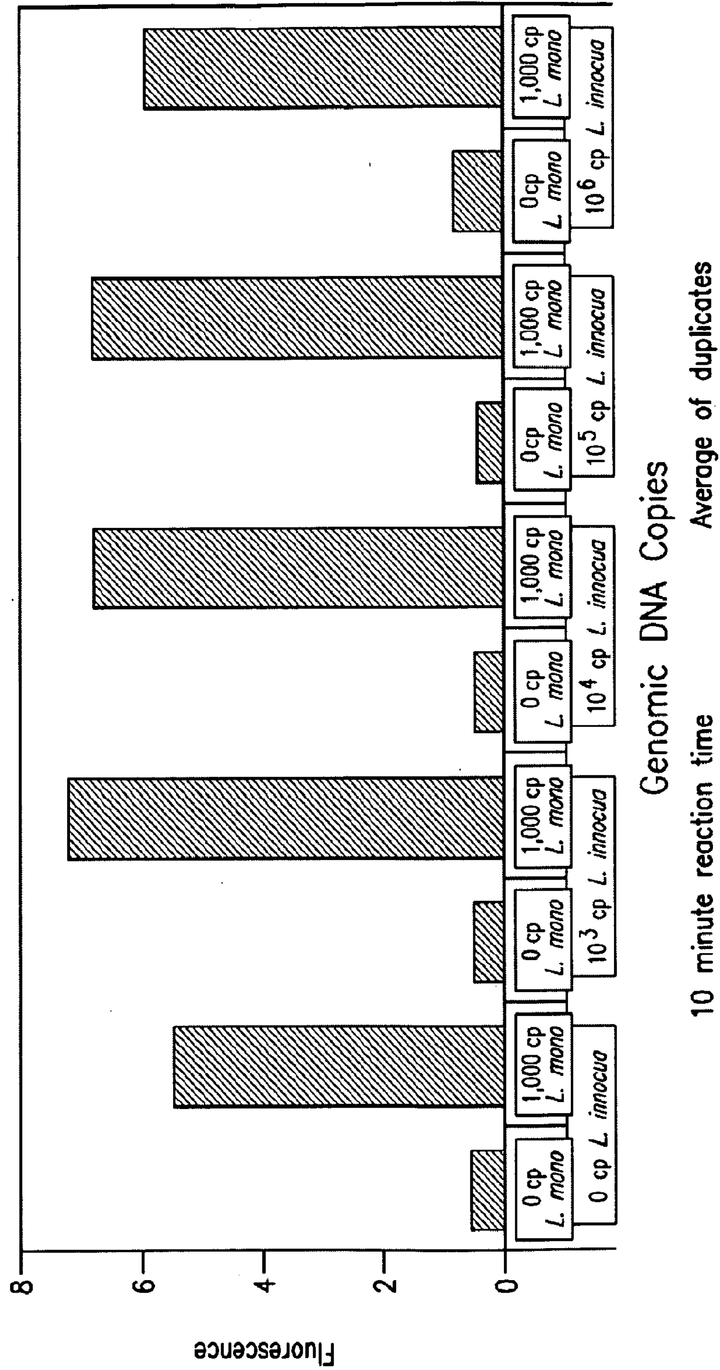


FIG.23

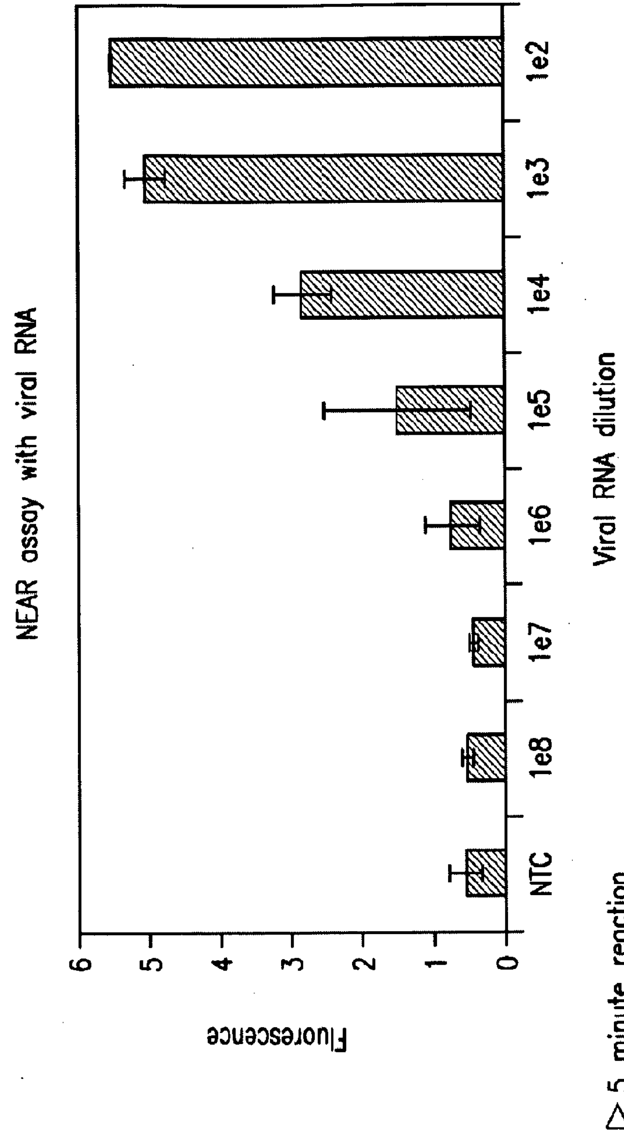


FIG.24

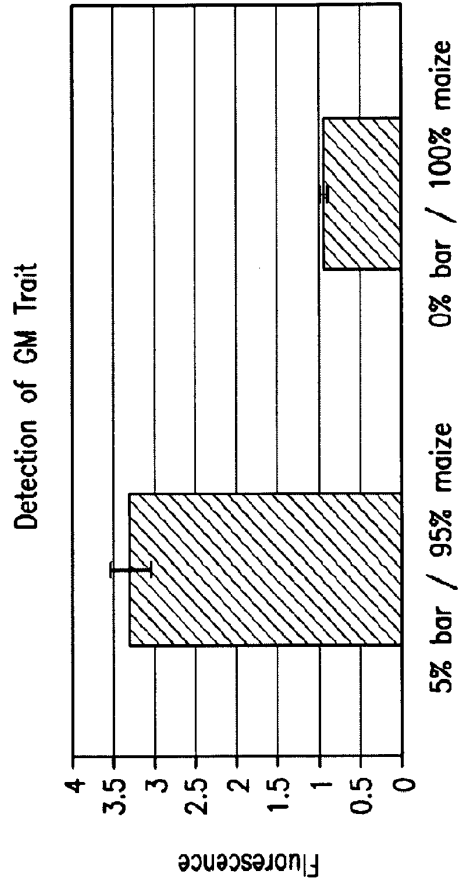
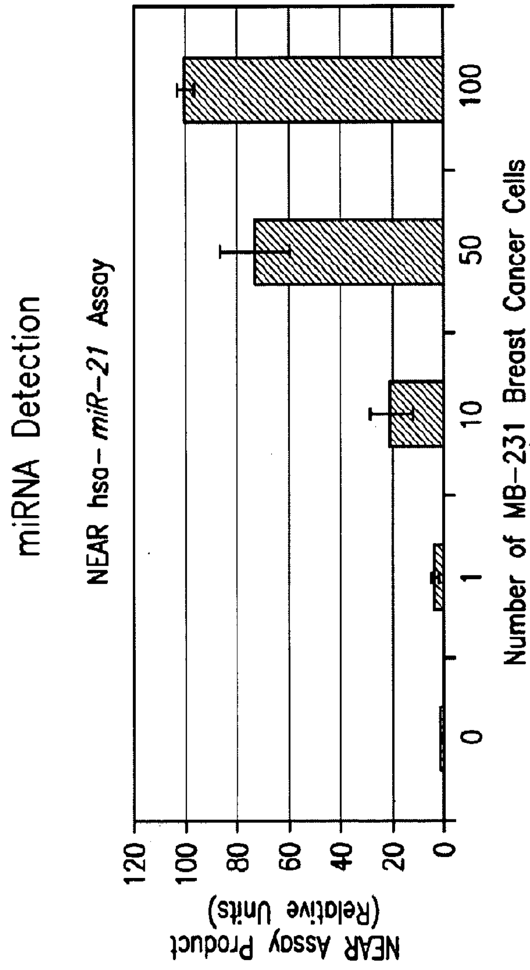


FIG.25



Number of cells based upon expected purified RNA yield per cell.
1ng RNA equates to ~100 cells

FIG.26

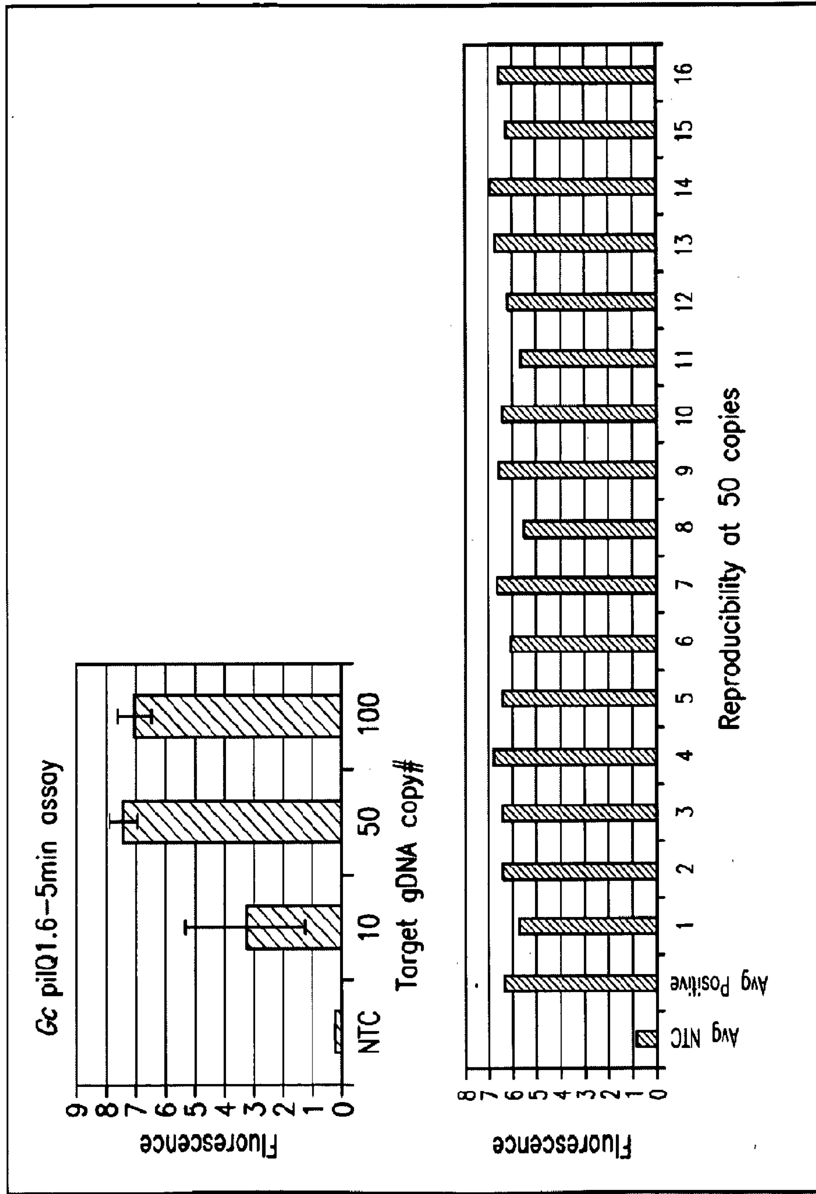


FIG.27

B. subtilis 1.25 NEAR Assay

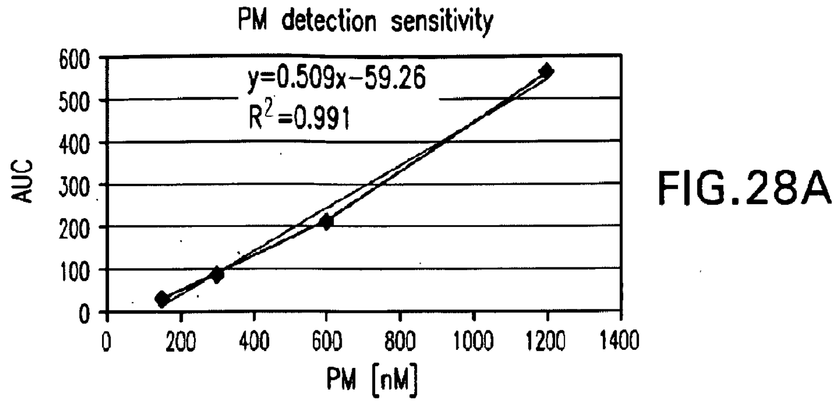


FIG.28A

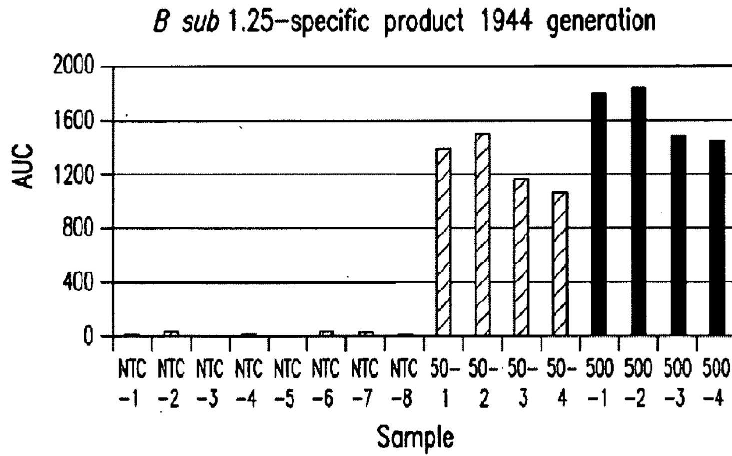


FIG.28B

Specific product 1944 yields (x=y-b/m)		
Sample	AUC signal	Product [nM]
50-1	1394	2851
50-2	1495	3049
50-3	1175	2421
50-4	1072	2219
500-1	1799	3645
500-2	1837	3720
500-3	1472	3004
500-4	1438	2937

FIG.28C

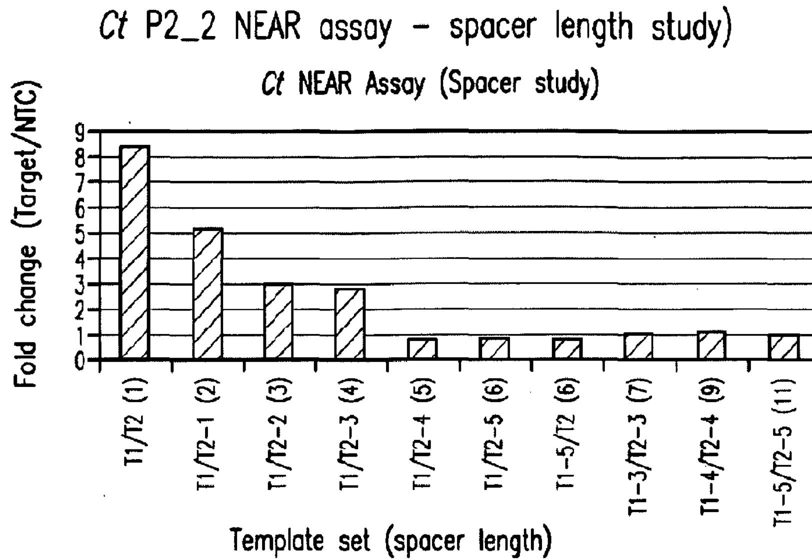


FIG. 29A

Temp1 12mer	ATGCATGCATGAGTCACATAGGCTTATGGAG
Temp1 -1 12mer	ATGCATGCATGAGTCACATgAGGCTTATGGA
Temp1 -2 12mer	ATGCATGCATGAGTCACATagAGGCTTATGG
Temp1 -3 12mer	ATGCATGCATGAGTCACATtagAGGCTTATG
Temp1 -4 12mer	ATGCATGCATGAGTCACATttagAGGCTTAT
Temp1 -5 12mer	ATGCATGCATGAGTCACATcttagAGGCTTA
Temp2 12mer	ATGCATGCATGAGTCACATTTATACCGCTTA
Temp2 -1 12mer	ATGCATGCATGAGTCACATtTTATACCGCTT
Temp2 -2 12mer	ATGCATGCATGAGTCACATttTTATACCGCT
Temp2 -3 12mer	ATGCATGCATGAGTCACATgttTTATACCGC
Temp2 -4 12mer	ATGCATGCATGAGTCACATtgttTTATACCG
Temp2 -5 12mer	ATGCATGCATGAGTCACATatgttTTATACC

FIG. 29B

Ct NEAR assay–Template designs to analyze effect of spacer length

Seq ID	Seq orientation	Sequence
T2-5	3'/5'	CCATATTttgtaTACACTGAGTACGTACGTA
T2-4	3'/5'	GCCATATTttgTACACTGAGTACGTACGTA
T2-3	3'/5'	CGCCATTttgTACACTGAGTACGTACGTA
T2-2	3'/5'	TCGCCATATTttTACACTGAGTACGTACGTA
T2-1	3'/5'	TTCGCCATATTtTACACTGAGTACGTACGTA
T2	3'/5'	ATTCGCCATATTTACACTGAGTACGTACGTA
Target-sense	5'/3'	cttagAGGCTTATGCAGTTAAGCGGTATAAaacat
Target-antisense	3'/5'	gaatcTCCGAATACCTCAATTCGCCATATTttgta
T1	5'/3'	ATGCATGCATGAGTCACATAGGCTTATGCAG
T1-1	5'/3'	ATGCATGCATGAGTCACATgAGGCTTATGCA
T1-2	5'/3'	ATGCATGCATGAGTCACATagAGGCTTATGC
T1-3	5'/3'	ATGCATGCATGAGTCACATtagAGGCTTATG
T1-4	5'/3'	ATGCATGCATGAGTCACATttagAGGCTTAT
T1-5	5'/3'	ATGCATGCATGAGTCACATcttagAGGCTTA

Example – 11mer spacer

Seq ID	Seq orientation	Sequence
T2-5	3'/5'	CCATATTttgtaTACACTGAGTACGTACGTA
Target-sense	5'/3'	cttagAGGCTTATGCAGTTAAGCGGTATAAaacat
Target-antisense	3'/5'	gaatcTCCGAATACCTCAATTCGCCATATTttgta
T1-5	5'/3'	ATGCATGCATGAGTCACATcttagAGGCTTA
Product (35mer)	5'/3'	cttagAGGCTTATGCAGTTAAGCGGTATAAaacat

FIG.30

Ct NEAR assay- requirement for template 'Stabilizing region'

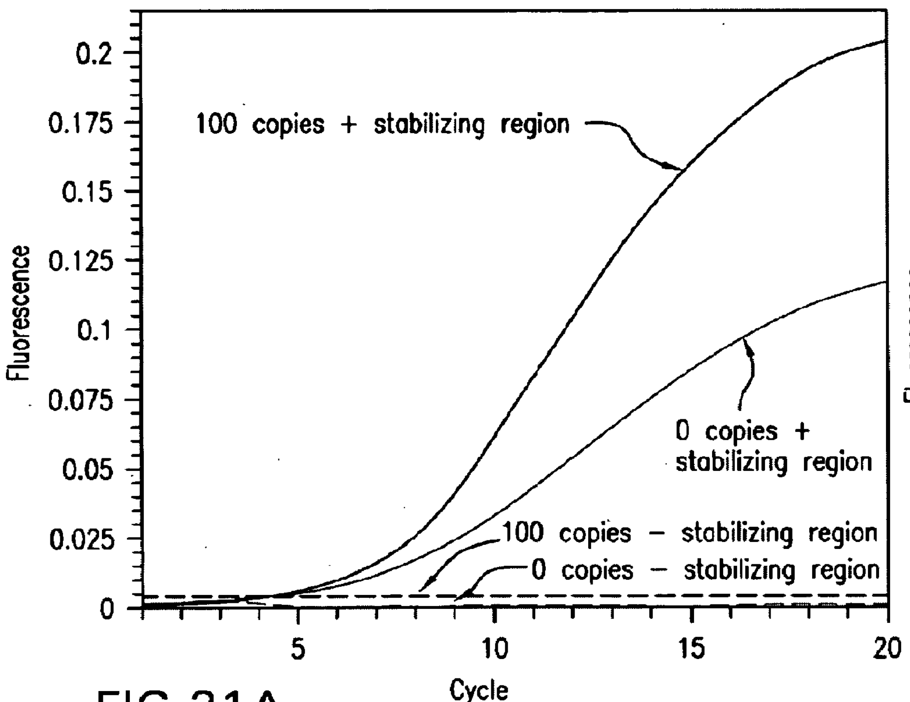


FIG.31A

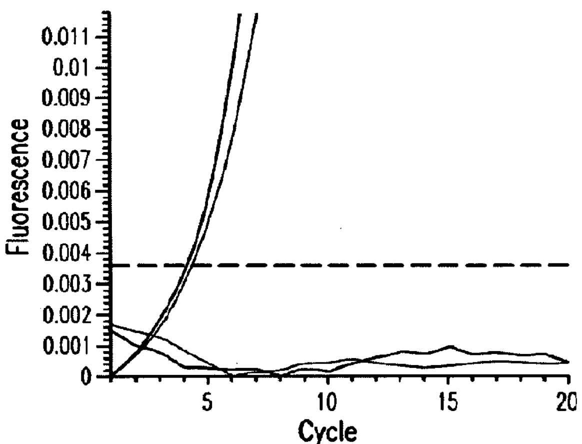


FIG.31B

Ct NEAR assay-Mg+2[] titration

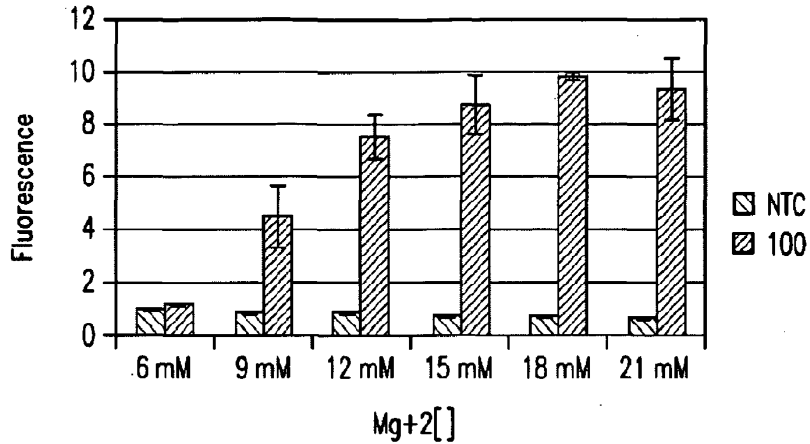


FIG.32A

Assay ID	*Ct* Ps_2	
Target	*Chlamydia trachomatis*	
	synthetic	
MB ID/[nM]	MB5.18/400	
Template ratio [nM]	200:100	
Replicates	2	
Experiment Date	1/14/2008	
Step	Time (min)	Temp (°C)
Reaction	5	56
Enzyme Inactivation	2	80
Readout	1	56
Comments: Comments: In-house buffer was used at 50mM Tris-HCl, pH8.6, 1mM DTT, 0.1% Tx-100		

FIG.32B

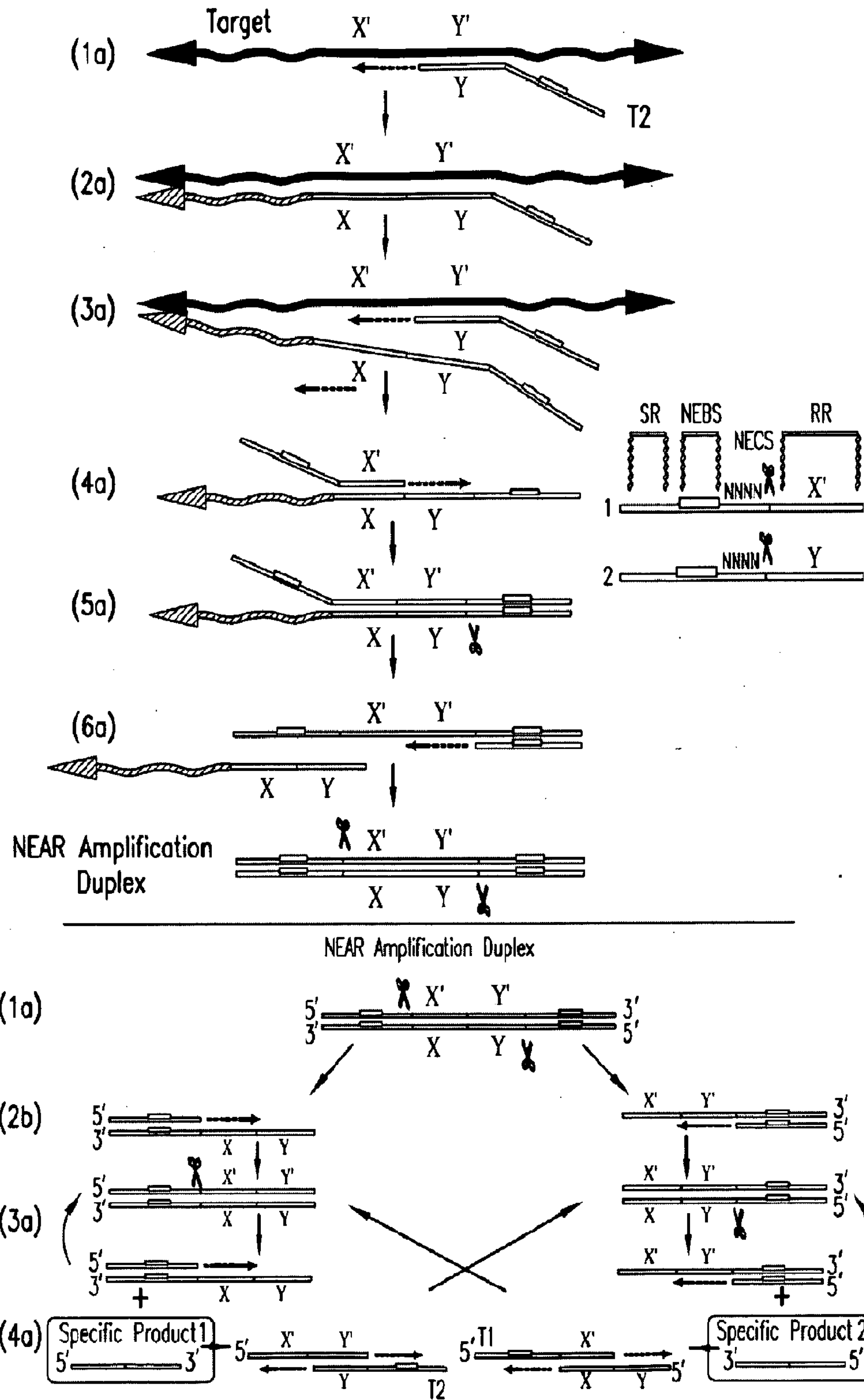


FIG.33

Target Organism	Target	Genome	Assay
Chlamydia trachomatis	Bacteria	DNA	P2_2
C trachomatis	Bacteria	RNA	P2_2
Neisseria gonorrhoeae	Bacteria	DNA	16S-4
N gonorrhoeae	Bacteria	DNA	pilQ 1.6
Mycobacterium tuberculosis	Bacteria	DNA	ITS.23s.5s.A.F/R1.12
Enterovirus	Virus	RNA	F8/R9.11
Clostridium difficile	Bacteria	DNA	TcdB F24/R25
C difficile	Bacteria	DNA	TcdB F25/R25
C difficile	Bacteria	DNA	TcdB F24/R24
Listeria monocytogenes	Bacteria	DNA	Lmono 579
Foot & Mouth disease virus	Virus	RNA	F1.2/R1.2
Foot & Mouth disease virus	Virus	RNA	F1.7/R1.7
Human miRNA	Eukaryote	RNA	miRNA 21
Human miRNA	Eukaryote	RNA	miRNA 335
Bacillus subtilis	Bacteria	DNA	ppsA 1.25
B subtilis	Bacteria	RNA	ppsA 1.25
Adenovirus 5	Virus	DNA	E1A 1.11
Methicillin-resistant Staphylococcus aureus	Bacteria	DNA	mecA 1359
MRSA	Bacteria	DNA	mecA 1520
MRSA	Bacteria	DNA	SA_nuc 355
MRSA	Bacteria	DNA	SA_nuc 368
MRSA	Bacteria	DNA	SA_nuc 662
Salmonella spp	Bacteria	DNA	spaO 4
Acinetobacter baumanii	Bacteria	DNA	A.ba.gyrB.A.12.F9/R9
Escherichia coli	Bacteria	DNA	Ecoli 4.F/R

FIG.34A

Template (5'-3')	Template 2 (5'-3')	Target (5'-3')
ATGCATGCATGAGTCACATAGGCTTATGGAG	ATGCATGCATGAGTCACATTTATACCGCTTA	AGGCTTATGGAGTTAAGCGGTATAA
ATGCATGCATGAGTCACATAGGCTTATGGAG	ATGCATGCATGAGTCACATTTATACCGCTTA	AGGCTTATGGAGTTAAGCGGTATAA
ATGCATGCATGAGTCACATCGCATACGTCTT	ATGCATGCATGAGTCACATCCTGCTTTCCCT	CGCATACGTCTTGAGAGGGAAAGCAGG
ATGCATGCATGAGTCACATTTTTTGAGTTCC	ATGCATGCATGAGTCACATACTCTACCAACA	ACTCTACCAACACGGAACTCAAAAA
ATGCATGCATGAGTCACATAAACAAACTCGC	ATGCATGCATGAGTCACATAACGGATGTGGT	AAACAAACTCGCAACCACATCCGTT
ATGCATGCATGAGTCACATCGACTACTTTGG	ATGCATGCATGAGTCACATGAAACACGGAC	CGACTACTTTGGGTGTCCGTGTTTC
ATGCATGCATGAGTCACATAGAAACTGGAGA	ATGCATGCATGAGTCACATCTACAAATATAG	AGAAACTGGAGAATCTATATTTGTAG
ATGCATGCATGAGTCACATGAAACTGGAGAA	ATGCATGCATGAGTCACATCTACAAATATAG	GAAACTGGAGAATCTATATTTGTAG
ATGCATGCATGAGTCACATAGAAACTGGAG	ATGCATGCATGAGTCACATCAAATATAGAT	AGAAACTGGAGAATCTATATTTG
ATGCATGCATGAGTCACATAAAGCAAGAGAA	ATGCATGCATGAGTCACATATACACGATAAC	AAAGCAAGAGAAAGTTATCGTGTAT
ATGCATGCATGAGTCACATAGGCTAAGGATG	ATGCATGCATGAGTCACATGGTACCTGAAGG	AGGCTAAGGATGCCCTTCAGGTACC
ATGCATGCATGAGTCACATGCCCTTCAGGTA	ATGCATGCATGAGTCACATTGTTACCTCGGG	GCCCTTCAGGTACCCCGAGGTAACA
ATGCATGCATGAGTCACATTAGCTTATCA	ATGCATGCATGAGTCACATTCAACATCAG	UAGCUUAUCAGACUGAUGUUGA
ATGCATGCATGAGTCACATTCAAGAGCAA	ATGCATGCATGAGTCACATACATTTTTCGT	UCAAGAGCAAUAACGAAAAAUGU
ATGCATGCATGAGTCACATCCAAGCTCAAAA	ATGCATGCATGAGTCACATTTCACGATTCCT	CCAAGCTCAAAAAAGGAATCGTGAA
ATGCATGCATGAGTCACATCCAAGCTCAAAA	ATGCATGCATGAGTCACATTTCACGATTCCT	CCAAGCTCAAAAAAGGAATCGTGAA
ATGCATGCATGAGTCACATCAAGACCTACC	ATGCATGCATGAGTCACATTTTAGGACGGC	CAAGACCTACCCGCCGTCCTAAA
ATGCATGCATGAGTCACATCGGTTTTAAGTG	ATGCATGCATGAGTCACATGATACCTTCGTT	GATACCTTCGTTCCACTTAAAACCG
ATGCATGCATGAGTCACATGCCAATTCCACA	ATGCATGCATGAGTCACATTAGACCGAAACA	GCCAATTCCACATTGTTTCGGTCTA
ATGCATGCATGAGTCACATGATACACCTGAA	ATGCATGCATGAGTCACATTAGGATGCTTTG	GATACACCTGAAACAAAGCATCCTA
ATGCATGCATGAGTCACATCAAAGCATCCTA	ATGCATGCATGAGTCACATTCTACACCTTTT	CAAAGCATCCTAAAAAAGGTGTAGA
ATGCATGCATGAGTCACATAAGACAACGCTG	ATGCATGCATGAGTCACATTATTGACCTGAA	AAGACAACGCTGATTCAGGTCAATA
ATGCATGCATGAGTCACATGAAATGACCTAA	ATGCATGCATGAGTCACATCTACGCGAAAAA	GAAATGACCTAACTTTTTCGCGTAG
ATGCATGCATGAGTCACATAAATTCTCGTCT	ATGCATGCATGAGTCACATTTTCTTTTGTCT	AAATTCTCGTCTCAGACAAAAGAAA
ATGCATGCATGAGTCACATAGTTTCGACTGT	ATGCATGCATGAGTCACATGTTAAGCAGGAA	AGTTTCGACTGTTTTCCTGCTTAAC

FIG.34B

EUROPEAN SEARCH REPORT

Application Number
EP 12 19 5331

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X	NESS VAN J ET AL: "Isothermal reactions for the amplification of oligonucleotides", PROCEEDINGS OF THE NATIONAL ACADEMY OF SCIENCES OF USA, NATIONAL ACADEMY OF SCIENCE, WASHINGTON, DC.; US, vol. 100, no. 8, 15 April 2003 (2003-04-15), pages 4504-4509, XP002326427, ISSN: 0027-8424 * the whole document *	1-22	TECHNICAL FIELDS SEARCHED (IPC) C12Q
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The present search report has been drawn up for all claims

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Place of search	Date of completion of the search	Examiner
The Hague	20 September 2013	Aguilera, Miguel

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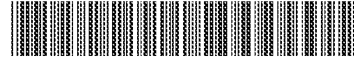

(11) **EP 2 660 333 A1**

(12) **EUROPEAN PATENT APPLICATION**

(43) Date of publication:
06.11.2013 Bulletin 2013/45

(51) Int Cl.:
C12Q 1/68 (2006.01)

(21) Application number: **12195333.5**

(22) Date of filing: **14.07.2008**

(84) Designated Contracting States:
AT BE BG CH CY CZ DE DK EE ES FI FR GB GR HR HU IE IS IT LI LT LU LV MC MT NL NO PL PT RO SE SI SK TR

(30) Priority: **14.07.2007 US 778018**

(62) Document number(s) of the earlier application(s) in accordance with Art. 76 EPC:
08781827.4 / 2 181 196

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Remarks:
•This application was filed on 03-12-2012 as a divisional application to the application mentioned under INID code 62.
•Claims filed after the date of filing of the application / after date of receiptof divisional application (Rule 68 (4) EPC).

(54) **Nicking and extension amplification reaction for the exponential amplification of nucleic acids**

(57) The invention is in general directed to the rapid exponential amplification of short DNA or RNA sequences at a constant temperature.

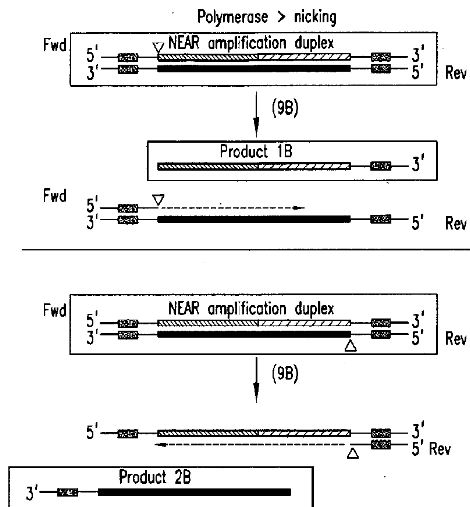

FIG.1C

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Description

Field of the Invention

5 **[0001]** The invention is in general directed to the rapid exponential amplification of short DNA or RNA sequences at a constant temperature.

Related Applications

10 **[0002]** Priority is claimed to U.S. Patent Application serial number 11/778,018, filed July 14, 2007, and entitled Nicking and Extension Amplification Reaction for the Exponential Amplification of Nucleic Acids, which is referred to and incorporated herein by reference in its entirety.

Background

15

[0003] The field of *in vitro* diagnostics is quickly expanding as the need for systems that can rapidly detect the presence of harmful species or determine the genetic sequence of a region of interest is increasing exponentially. Current molecular diagnostics focus on the detection of biomarkers and include small molecule detection, immuno-based assays, and nucleic acid tests. The built-in specificity between two complementary or substantially complementary nucleic acid 20 strands allows for fast and specific recognition using unique DNA or RNA sequences, the simplicity of which makes a nucleic acid test an attractive prospect. Identification of bacterial and viral threat agents, genetically modified food products, and single nucleotide polymorphisms for disease management are only a few areas where the advancement of these molecular diagnostic tools becomes extremely advantageous. To meet these growing needs, nucleic acid amplification technologies have been developed and tailored to these needs of specificity and sensitivity.

25 **[0004]** Historically, the most common amplification technique is the polymerase chain reaction (PCR), which has in many cases become the gold standard for detection methods because of its reliability and specificity. This technique requires the cycling of temperatures to proceed through the steps of denaturation of the dsDNA, annealing of short oligonucleotide primers, and extension of the primer along the template by a thermostable polymerase. Though many new advances in engineering have successfully shortened these reaction times to 20-30 minutes, there is still a steep 30 power requirement to meet the needs of these thermocycling units.

[0005] Various isothermal amplification techniques have been developed to circumvent the need for temperature cycling. From this demand, both DNA and RNA isothermal amplification technologies have emerged.

[0006] Transcription-Mediated Amplification (TMA) employs a reverse transcriptase with RNase activity, an RNA polymerase, and primers with a promoter sequence at the 5'end. The reverse transcriptase synthesizes cDNA from the 35 primer, degrades the RNA target, and synthesizes the second strand after the reverse primer binds. RNA polymerase then binds to the promoter region of the dsDNA and transcribes new RNA transcripts which can serve as templates for further reverse transcription. The reaction can produce a billion fold amplification in 20-30 minutes. This system is not as robust as other DNA amplification techniques and is therefore, not a field-deployable test due to the ubiquitous presence of RNAases outside of a sterile laboratory. This amplification technique is very similar to Self-Sustained 40 Sequence Replication (3 SR) and Nucleic Acid Sequence Based Amplification (NASBA), but varies in the enzymes employed.

[0007] Single Primer Isothermal Amplification (SPIA) also involves multiple polymerases and RNaseH. First, a reverse transcriptase extends a chimeric primer along an RNA target. RNaseH degrades the RNA target and allows a DNA polymerase to synthesize the second strand of cDNA. RNaseH then degrades a portion of the chimeric primer to release 45 a portion of the cDNA and open a binding site for the next chimeric primer to bind and the amplification process proceeds through the cycle again. The linear amplification system can amplify very low levels of RNA target in roughly 3.5 hrs.

[0008] The Q-Beta replicase system is a probe amplification method. A probe region complementary or substantially complementary to the target of choice is inserted into MDV-1 RNA, a naturally occurring template for Q-Beta replicase. Q-Beta replicates the MDV-1 plasmid so that the synthesized product is itself a template for Q-Beta replicase, resulting 50 in exponential amplification as long as the there is excess replicase to template. Because the Q-Beta replication process is so sensitive and can amplify whether the target is present or not, multiple wash steps are required to purge the sample of non-specifically bound replication plasmids. The exponential amplification takes approximately 30 minutes; however, the total time including all wash steps is approximately 4 hours.

[0009] Numerous isothermal DNA amplification technologies have been developed as well. Rolling circle amplification 55 (RCA) was developed based on the natural replication of plasmids and viruses. A primer extends along a circular template resulting in the synthesis of a single-stranded tandem repeat. Capture, washing, and ligation steps are necessary to preferentially circularize the template in the presence of target and reduce background amplification. Ramification amplification (RAM) adds cascading primers for additional geometric amplification. This technique involves amplification of

non-specifically sized strands that are either double or single-stranded.

[0010] Helicase-dependent amplification (HDA) takes advantage of a thermostable helicase (Tte-UvrD) to unwind dsDNA to create single-strands that are then available for hybridization and extension of primers by polymerase. The thermostable HDA method does not require the accessory proteins that the non-thermostable HDA requires. The reaction can be performed at a single temperature, though an initial heat denaturation to bind the primers generates more product. Reaction times are reported to be over 1 hour to amplify products 70-120 base pairs in length.

[0011] Loop mediated amplification (LAMP) is a sensitive and specific isothermal amplification method that employs a thermostable polymerase with strand displacement capabilities and four or more primers. The primers are designed to anneal consecutively along the target in the forward and reverse direction. Extension of the outer primers displaces the extended inner primers to release single strands. Each primer is designed to have hairpin ends that, once displaced, snap into a hairpin to facilitate self-priming and further polymerase extension. Additional loop primers can decrease the amplification time, but complicates the reaction mixture. Overall, LAMP is a difficult amplification method to multiplex, that is, to amplify more than one target sequence at a time, although it is reported to be extremely specific due to the multiple primers that must anneal to the target to further the amplification process. Though the reaction proceeds under isothermal conditions, an initial heat denaturation step is required for double-stranded targets. Amplification proceeds in 25 to 50 minutes and yields a ladder pattern of various length products.

[0012] Strand displacement amplification (SDA) was developed by Walker et.al. in 1992. This amplification method uses two sets of primers, a strand displacing polymerase, and a restriction endonuclease. The bumper primers serve to displace the initially extended primers to create a single-strand for the next primer to bind. A restriction site is present in the 5' region of the primer. Thiol-modified nucleotides are incorporated into the synthesized products to inhibit cleavage of the synthesized strand. This modification creates a nick site on the primer side of the strand, which the polymerase can extend. This approach requires an initial heat denaturation step for double-stranded targets. The reaction is then run at a temperature below the melting temperature of the double-stranded target region. Products 60 to 100 bases in length are usually amplified in 30-45 minutes using this method.

[0013] These and other amplification methods are discussed in, for example, VanNess, J, et al., PNAS 2003, vol 100, no 8, p 4504-4509; Tan, E., et al., Anal. Chem. 2005, 77, 7984-7992; Lizard, P., et al., Nature Biotech. 1998, 6, 1197-1202; Notomi, T., et al., NAR 2000, 28, 12, e63; and Kurn, N., et al., Clin. Chem. 2005, 51:10, 1973-1981. Other references for these general amplification techniques include, for example, U.S. Patent Serial Nos. 7112423; 5455166; 5712124; 5744311; 5916779; 5556751; 5733733; 5834202; 5354668; 5591609; 5614389; 5942391; and U.S. patent publication numbers US20030082590; US20030138800; US20040058378; and US20060154286.

Summary

[0014] Provided herein are methods of amplifying nucleic acid target sequences that rely on nicking and extension reactions to amplify shorter sequences in a quicker timeframe than traditional amplification reactions, such as, for example, strand displacement amplification reactions. Embodiments of the invention include, for example, reactions that use only two templates to amplify a target sequence, one or two nicking enzymes, and a polymerase, under isothermal conditions. In exemplary embodiments, the polymerase and the nicking enzyme are thermophilic, and the reaction temperature is significantly below the melting temperature of the hybridized target region. The nicking enzyme nicks only one strand in a double-stranded duplex, so that incorporation of modified nucleotides is not necessary as in the case of conventional strand displacement amplification. An initial heat denaturation step is not required for the methods of the present invention. Due to the simplicity of the reaction, in exemplary embodiments, the reaction is very easy to perform, requires no special equipment, such as a thermocycler, and can amplify 20-30mer products 10^8 to 10^{10} fold from genomic DNA in only about 2.5 to about 10 minutes. Furthermore, in other exemplary embodiments, the method is able to amplify RNA without a separate reverse transcription step.

[0015] Thus, provided in a first embodiment of the present invention is a method for amplifying a double-stranded nucleic acid target sequence, comprising contacting a target DNA molecule comprising a double-stranded target sequence having a sense strand and an antisense strand, with a forward template and a reverse template, wherein said forward template comprises a nucleic acid sequence comprising a recognition region at the 3' end that is complementary or substantially complementary to the 3' end of the target sequence antisense strand; a nicking enzyme binding site and a nicking site upstream of said recognition region, and a stabilizing region upstream of said nicking enzyme binding site and said nicking site; said reverse template comprises a nucleotide sequence comprising a recognition region at the 3' end that is complementary or substantially complementary to the 3' end of the target sequence sense strand, a nicking enzyme binding site and a nicking site upstream of said recognition region, and a stabilizing region upstream of said nicking enzyme binding site and said nicking site; providing a first nicking enzyme that is capable of nicking at the nicking site of said forward template, and does not nick within said target sequence; providing a second nicking enzyme that is capable of nicking at the nicking site of said reverse template and does not nick within said target sequence; and providing a DNA polymerase; under conditions wherein amplification is performed by multiple cycles of said polymerase extending

said forward and reverse templates along said target sequence producing a double-stranded nicking site, and said nicking enzymes nicking at said nicking sites, producing an amplification product.

[0016] In certain embodiments of the invention, the DNA polymerase is a thermophilic polymerase. In other examples of the invention, the polymerase and said nicking enzymes are stable at temperatures up to 37°C, 42°C, 60°C, 65°C, 70°C, 75°C, 80°C, or 85°C. In certain embodiments, the polymerase is stable up to 60°C. The polymerase may, for example, be selected from the group consisting of Bst (large fragment), 9°N, Vent$_R$® (exo-) DNA Polymerase, Therminator, and Therminator II.

[0017] The nicking enzyme may, for example, nick upstream of the nicking enzyme binding site, or, in exemplary embodiments, the nicking enzyme may nick downstream of the nicking enzyme binding site. In certain embodiments, the forward and reverse templates comprise nicking sites recognized by the same nicking enzyme and said first and said second nicking enzyme are the same. The nicking enzyme may, for example, be selected from the group consisting of Nt.BspQI, Nb.BbvCi, Nb.BsmI, Nb.BsrDI, Nb.BtsI, Nt.AlwI, Nt.BbvCI, Nt.BstNBI, Nt.CviPII, Nb.Bpu10I, and Nt.Bpu10I.

[0018] In certain aspects of the present invention, the target sequence comprises from 1 to 5 nucleotides more than the sum of the nucleotides of said forward template recognition region and said reverse template recognition region.

[0019] The DNA molecule may be, for example, genomic DNA. The DNA molecule may be, for example, selected from the group consisting of plasmid, mitochondrial, and viral DNA. In certain embodiments, the forward template is provided at the same concentration as the reverse template. In other examples, the forward template is provided at a ratio to the reverse template at the range of ratios of 1:100 to 100:1.

[0020] In other examples of the invention, the method further comprises the use of a second polymerase. The amplification may be, for example, conducted at a constant temperature. This temperature may be, for example, between 54°C and 60°C. As to the length of time for the reaction to take place, in certain examples, the amplification reaction is held at constant temperature for 1 to 10 minutes.

[0021] The present invention further comprises detecting the amplification product, for example, by a method selected from the group consisting of gel electrophoresis, mass spectrometry, SYBR I fluorescence, SYBR II fluorescence, SYBR Gold, Pico Green, TOTO-3, intercalating dye detection, FRET, molecular beacon detection, surface capture, capillary electrophoresis, incorporation of labeled nucleotides to allow detection by capture, fluorescence polarization, and lateral flow capture. The amplification products may be, for example, detected using a solid surface method, for example, where at least one capture probe is immobilized on the solid surface that binds to the amplified sequence.

[0022] The present invention may be used for multiplex amplification. Thus, for example, in certain embodiments of the present invention at least two target sequences are capable of being amplified. By "capable of being amplified" is meant the amplification reaction comprises the appropriate templates and enzymes to amplify at least two target sequences. Thus, for example, the amplification reaction may be prepared to detect at least two target sequences, but only one of the target sequences may actually be present in the sample being tested, such that both sequences are capable of being amplified, even though only one sequence may actually be amplified. Or, where two target sequences are present, the amplification reaction may result in the amplification of both of the target sequences. The multiplex amplification reaction may result in the amplification of one, some, or all, of the target sequences for which it comprises the appropriate templates and enzymes.

[0023] At least one of the templates, for example, may comprise a spacer, a blocking group, or a modified nucleotide.

[0024] Also provided as an embodiment of the present invention is a method for amplifying a single-stranded nucleic acid target sequence, comprising contacting a target nucleic acid comprising a single-stranded target sequence with a reverse template, wherein said reverse template comprises a nucleotide sequence comprising a recognition region at the 3' end that is complementary or substantially complementary to the 3' end of the target sequence, a nicking enzyme binding site and a nicking site upstream of said recognition region, and a stabilizing region upstream of said nicking enzyme binding site and said nicking site; providing a first nicking enzyme that is capable of nicking at the nicking site of said reverse template, and does not nick within said target sequence; providing a DNA polymerase under conditions wherein said polymerase extends said reverse template along said target sequence; contacting said extended reverse template with a forward template, wherein said forward template comprises a recognition region at the 3' end that is identical to the 5' end of the target sequence a nicking enzyme binding site and a nicking site upstream of said recognition region, and a stabilizing region upstream of said nicking enzyme binding site and said nicking site; providing a second nicking enzyme that is capable of nicking at the nicking site of said forward template and does not nick within said target sequence; under conditions wherein amplification is performed by multiple cycles of said polymerase extending said forward and reverse templates along said target sequence producing a double-stranded nicking site, and said nicking enzymes nicking at said nicking sites, producing an amplification product.

[0025] Those of ordinary skill in the art understand that the examples presented herein relating to the amplification of a double-stranded nucleic acid target sequence and the detection of the amplified product also apply to the amplification of a single-stranded nucleic acid target sequence and the detection of the amplified product. Furthermore, in examples of the present invention, the target sequence may be, for example, RNA, for example, but not limited to, messenger RNA (mRNA), ribosomal RNA (rRNA), viral RNA, microRNA, a microRNA precursor, or siRNA. In exemplary embodiments

of the present invention, for example, where the target sequence is RNA, the polymerase has reverse transcription activity. In yet other examples of the present invention, the target sequence is DNA, such as, for example, genomic DNA, or for example, the target sequence is selected from the group consisting of plasmid, mitochondrial, and viral DNA, or even a PCR product.

[0026] Where the method, in accordance with the present invention, involves the use of more than one polymerase, in exemplary embodiments at least one of the polymerases may have reverse transcriptase activity.

[0027] In other embodiments of the present invention, a set of oligonucleotide templates is provided, comprising a first template for nucleic acid amplification, comprising a recognition region at the 3' end that is complementary or substantially complementary to the 3' end of a target sequence antisense strand; a nicking enzyme binding site and a nicking site upstream of said recognition region; and a stabilizing region upstream of said nicking enzyme binding site and said nicking site; and a second template for nucleic acid amplification, comprising a recognition region at the 3' end that is identical to the 5' of said target sequence antisense strand; nicking enzyme binding site and a nicking site upstream of said recognition region; and a stabilizing region upstream of said nicking enzyme binding site and said nicking site; wherein said target sequence comprises from 1 to 5 spacer bases between said 3' end of the antisense strand and said 5' end of said antisense strand that do not bind to either template.

[0028] In yet other embodiments, a kit is provided for following the methods of the present invention for nucleic acid amplification, comprising a DNA polymerase; a first template for nucleic acid amplification, comprising a recognition region at the 3' end that is complementary or substantially complementary to the 3' end of a target sequence antisense strand; a nicking enzyme binding site and a nicking site upstream of said recognition region; and a stabilizing region upstream of said nicking enzyme binding site and said nicking site; a second template for nucleic acid amplification, comprising a recognition region at the 3' end that is complementary or substantially complementary to the 3' end of a target sequence sense strand; nicking enzyme binding site and a nicking site upstream of said recognition region; and a stabilizing region upstream of said nicking enzyme binding site and said nicking site; one or two thermostable nicking enzymes, wherein either one enzyme is capable of nicking at the nicking site of said first and said second templates, or a first enzyme is capable of nicking at the nicking site of said first primer and a second enzyme is capable of nicking at the enzyme site of said second primer.

[0029] The kit may, for example, provide said polymerase, nicking enzymes, and templates in a container. The kit may provide, for example, said polymerase, nicking enzymes, and templates in two containers. In certain examples, the polymerase and nicking enzymes are in a first container, and said templates are in a second container. In certain examples, the polymerase and nicking enzymes are lyophilized. The kit may, for example, further comprise instructions for following the amplification methods of the present invention. The kit may, for example, further comprise a cuvette. The kit may, for example, further comprise a lateral flow device or dipstick. The lateral flow device or dipstick may, for example, further comprise a capture probe, wherein said capture probe binds to amplified product. The kit may, for example, further comprise a detector component, for example, one selected from the group consisting of a fluorescent dye, colloidal gold particles, latex particles, a molecular beacon, polystyrene beads, and the like. In other examples, at least one of the templates of the kit may comprise a spacer, blocking group, or a modified nucleotide.

[0030] Deoxynucleoside triphosphates (dNTPs) are included in the amplification reaction. One or more of the dNTPs may be modified, or labeled, as discussed herein, however, the use of modified NTPs is not required in the present method. Nucleotides are designated as follows. A ribonucleoside triphosphate is referred to as NTP or rNTP; wherein N can be A, G, C, U or m5U to denote specific ribonucleotides. Deoxynucleoside triphosphate substrates are indicated as dNTPs, wherein N can be A, G, C, T, or U. Throughout the text, monomeric nucleotide subunits may be denoted as A, G, C, or T with no particular reference to DNA or RNA.

[0031] In another embodiment, a method is provided for nucleic acid amplification comprising forming a mixture of a target nucleic acid comprising a double-stranded target sequence having a sense strand and an antisense strand; a forward template comprising a nucleic acid sequence comprising a recognition region at the 3' end that is complementary or substantially complementary to the 3' end of the target sequence antisense strand; a nicking enzyme binding site and a nicking site upstream of said recognition region, and a stabilizing region upstream of said nicking enzyme binding site and said nicking site; a reverse template comprising a nucleotide sequence comprising a recognition region at the 3' end that is complementary or substantially complementary to the 3' end of the target sequence sense strand, a nicking enzyme binding site and a nicking site upstream of said recognition region and a stabilizing region upstream of said nicking enzyme binding site and said nicking site; a first nicking enzyme that is capable of nicking at the nicking site of said forward template, and does not nick within said target sequence; a second nicking enzyme that is capable of nicking at the nicking site of said reverse template and does not nick within said target sequence; and a thermophilic polymerase under conditions wherein amplification is performed by multiple cycles of said polymerase extending said forward and reverse templates along said target sequence producing a double-stranded nicking site, and said nicking enzymes nicking at said nicking sites, producing an amplification product. In certain embodiments, the nicking enzyme binding sites on the forward and reverse templates are recognized by the same nicking enzyme, and only one nicking enzyme is used for the reaction.

[0032] In another embodiment, a method is provided for nucleic acid amplification comprising forming a mixture of a target nucleic acid comprising a single-stranded target sequence; a reverse template, wherein said reverse template comprises a nucleotide sequence comprising a recognition region at the 3' end that is complementary or substantially complementary to the 3' end of the target sequence, a nicking enzyme binding site and a nicking site upstream of said recognition region, and a stabilizing region upstream of said nicking enzyme binding site and said nicking site; a first nicking enzyme that is capable of nicking at the nicking site of said reverse template, and does not nick within said target sequence; a thermophilic polymerase under conditions wherein said polymerase extends said reverse template along said target sequence; a forward template, wherein said forward template comprises a nucleic acid sequence comprising a recognition region at the 3' end that is identical or substantially identical to the 5'end of the target sequence; and a second nicking enzyme that is capable of nicking at the nicking site of said forward template and does not nick within said target sequence; under conditions wherein amplification is performed by multiple cycles of said polymerase extending said forward and reverse templates along said target sequence producing a double-stranded nicking site, and said nicking enzymes nicking at said nicking sites, producing an amplification product. In certain embodiments, the nicking enzyme binding sites on the forward and reverse templates are recognized by the same nicking enzyme, and only one nicking enzyme is used for the reaction.

[0033] In other embodiments of the invention are provided methods for the separation of amplified nucleic acids obtained by the amplification methods of the invention. In yet further embodiments of the invention are provided methods for detecting and/or analyzing the amplified nucleic acids obtained by the amplification methods of the invention, including, for example, methods using SYBR I, II, SYBR Gold, Pico Green, TOTO-3, and most intercalating dyes, molecular beacons, FRET, surface capture using immobilized probes with fluorescence, electrochemical, or colorimetric detection, mass spectrometry, capillary electrophoresis, the incorporation of labeled nucleotides to allow detection by capture or fluorescence polarization, lateral flow, and other methods involving capture probes.

[0034] Methods using capture probes for detection include, for example, the use of a nucleic acid molecule (the capture probe) comprising a sequence that is complementary to, or substantially complementary to, an amplification product strand such that the capture probe binds to amplified nucleic acid. The probe may be linked to a detectable label in certain embodiments, and amplification product may be detected based on the detectable label of the probe specifically hybridized to the amplification product. The reaction may, for example, further comprise an antibody directed against a molecule incorporated into or attached to the capture probe. Or, for example, the capture probe, or a molecule that binds to the capture probe, may incorporate, for example, an enzyme label, for example, peroxidase, alkaline phosphatase, or beta-galactosidase, a fluorescent label, such as, for example, fluorescein or rhodamine, or, for example, other molecules having chemiluminescent or bioluminescent activity. In some embodiments, the probe is linked to a solid support, and amplification product strands may be specifically immobilized to the capture probe linked to the solid support under conditions known and selected by the person of ordinary skill in the art. In the latter embodiments, solid support-immobilized amplification product may be subjected to processing steps, such as washing, ion exchange, release from the solid support, or other processing steps. An amplification product may be detected when immobilized to a solid support in some embodiments. The embodiments of the present invention also comprise combinations of these detection and analysis methods.

Brief Description of the Drawings

[0035]

Figures 1A-D are graphic drawings depicting mechanisms of the reactions of the present invention. Figure 1D is a legend for Figure 1.

Figure 2. 20% polyacrylamide gel of reaction products from a DNA NEAR™ assay.

A reaction following the present methods was run for 2.5 minutes at 56 °C, then heat denatured at 94 °C for 4 minutes. Six microliters of the reaction was run on a 20% polyacrylamide gel at 160V for ~2.5 hrs. The gel was stained with SYBR II gel stain. Lane 1: no target control for 25mer assay. Lane 2: no target control for 27mer assay. Lane 3: for 25mer assay with 3.5E+5 copies of genomic *Bacillus subtilis* DNA. Lane 4: for 27mer assay with 1.1E+6 copies of genomic *Bacillus subtilis* DNA.

Figure 3. 20% polyacrylamide gel of reaction products from an RNA assay using the present methods.

The reaction was run for 12 minutes at 56 °C, then heat denatured at 94 °C for 4 minutes. Six microliters of the reaction was run on a 20% polyacrylamide gel at 160V for about 2.5 hrs. The gel was stained with SYBR II gel stain. Lane 1 and 2: reaction for 25mer assay with 1E+6 copies of Ebola Armored RNA (Ambion). Lane 3 and 4: reaction no target control for 25mer assay. 25mer reaction products are outlined in the white box.

Figure 4. Mass Spectrum of *Bacillus anthracis* DNA assay products.

A) 0 copies of target or B) 5E+5 copies of genomic DNA added to the reaction. The reaction was run for 10

minutes, then heat denatured at 94 °C for 4 minutes. Ten microliters of sample was injected into the LC/ESI-MS. The (-4) charge state of the 26mer product and its complementary sequence are outlined in a black box. The smaller adjacent peaks are the sodium adducts of the main product.

5 **Figure 5.** Mass Spectrum of MS2 genomic RNA assay products.

A) 0 copies of target, B) 1E+6 copies of MS2 genomic RNA, or C) 1E+6 copies of synthetic target DNA added to the reaction. The reaction was run for 10 minutes, then heat denatured at 94 °C for 4 minutes. Ten microliters of sample was injected into the LC/ESI-MS. The (-4) charge state of the 27mer product and its complement
10 sequence are outlined in a black box. The smaller adjacent peaks are the sodium adducts of the main product.

Figure 6. Real-time detection of amplification using intercalating fluorescent dyes.
Real-time amplification of *Yersinia pestis* genomic DNA at 500 copies (squares) compared to the no target control (NTC, open triangles). The reaction was run for 10 minutes at 58 °C and monitored by the real-time fluorescence
15 with SYBR II (n = 5).
Figure 7. Real-time detection of amplification using fluorescence resonance energy transfer (FRET).
Real-time amplification of *Yersinia pestis* synthetic DNA at 10,000 copies (squares) compared to the no target control (NTC, open triangles). The reaction was run for 10 minutes at 57 °C, n = 3.
Figure 8. Francisella *tularensis* DNA amplification detected in real-time using molecular beacons.
20 Either 0 copies (open triangles) or 1E+5 copies (squares) were added to the reaction mix and run for 10 minutes at 57.5 °C.
Figure 9. False positive rate testing results comparing average AUC values.
Error bars denote one standard deviation. *Bacillus subtilis* assays were run for 10 min at 55 °C in the presence and absence of *Bacillus subtilis* genomic DNA. Enzymes were heat denatured at 94 °C for 4 min. A 10 μL sample was
25 injected into the LC/ESI-MS and the area under the curve (AUC) of the product peaks were analyzed. True Positives contained 10,000 copies of *Bacillus subtilis* DNA along with 990,000 copies of near neighbor (*Bacillus thuringiensis*) DNA. True Negatives contained 10,000 copies of *E. coli* DNA with 990,000 copies of near neighbor DNA, and water negatives contained no DNA as a control.
Figure 10. Replication study using molecular beacon detection with different operators performing the experiments
30 on two different days.
The reaction was run for 10 minutes at 57.5 °C (in the presence and absence of 500 copies of *Francisella tularensis* genomic DNA) with a 4 min heat kill at 94 °C. 300nM molecular beacon was added and monitored at 45, 50, and 57 °C (n = 24).
Figure 11. Sensitivity of the reaction using molecular beacon detection.
35 The assay was run for 10 minutes 57.5 °C. The reaction was stopped with a 4 min heat inactivation step at 94 °C. 300nM molecular beacon was added and the fluorescence was monitored at 57.5 °C (n = 3). Fluorescence was monitored for beacon opening in the presence of reactions amplified with 1E+6, 5E+5, 5E+4, 5E+2, 50, and 0 (NTC) input copies of *Francisella tularensis* genomic DNA, and compared to the background fluorescence of the beacon alone (MB).
40 **Figure 12.** Final concentration of amplified products in the NEAR reaction.
The NEAR™ reaction was run for 10 min at 55 °C with varying copies of *Bacillus subtilis* genomic DNA. The reaction was stopped with a heat inactivation step at 94 °C for 4 minutes. A 10 microliter sample was injected into the LC/ESI-MS and the AUC of the product peak at 1944 Daltons was analyzed and compared to a standard curve.
Figure 13. Correlation of the input RNA target copy number to the final concentration of amplified products.
45 The Ebola NEAR ™ assay was run for 12 min at 55 °C with varying copies of synthetic RNA corresponding to the Ebola genome DNA. The reaction was stopped with a heat inactivation step at 94 °C for 4 minutes. A 10 microliter sample was injected into the LC/ESI-MS and the AUC of the product peak at 1936 Daltons was analyzed and compared to the standard curve of AUC values. (n = 3)
Figure 14. Mass spec product analysis demonstrating NEAR reaction specificity.
50 The *Bacillus anthracis* NEAR™ reaction was run in the presence of a dilution of copies of Bacillus thuringiensis for 10 min at 56 °C (n = 3), then heat denatured at 94°C for 4 minutes. A 10 μL sample was injected into the LC/ESI-MS and AUC values of product peaks analyzed.
Figure 15. The effect of an interferent panel on amplification.
Bacillus subtilis DNA reactions were run for 10 min at 55 °C and heated to 94 °C for 4 minutes to stop the reaction.
55 Reactions were run in triplicate in the presence 1E+5 copies of *Bacillus subtilis* genomic DNA ("_1E+5") or with no target DNA present ("_0"). Sample x is the control assay with no interferent added. Interferents A through F were added at 50% reaction volume to the *Bacillus subtilis* assay. The AUC of mass spec product peaks were analyzed using a two-way ANOVA and Bonferroni t-test. (Key: A = none; B = House dust, skim milk; C = AZ test dust, humic

acid; D = Diesel soot; E = Skim milk; F = Mold spores)

Figure 16. Gel electrophoresis results for the *Bacillus subtilis / Bacillus anthracis* DNA duplex reaction.

The NEAR™ reaction including templates specific for both *Bacillus subtilis (Bs)* and *Bacillus anthracis (Ba)* DNA was run in the absence of target DNA (negative), in the presence of *Bacillus subtilis* only (positive for 27mer product), and in the presence of both *Bacillus subtilis* and *Bacillus anthracis* (positive for 27mer and 25mer product respectively). The target copy number used in this assay was 500,000 copies. The assay was run for 10 min at 57 °C. Templates varied in concentration between the assays to control the amplification (100nM for *Bacillus anthracis* and 50 nM for *Bacillus subtilis)*. Samples were run on a 20% polyacrylamide gel at 160 V for about 2 hours. The gel was stained with SYBR II fluorescent dye and imaged. The fluorescent bands were quantified and analyzed as the integrated optical density (IOD) (n = 8).

Figure 17. Specificity results for the *Bacillus subtilis / Bacillus anthracis* DNA duplex reaction shown by gel electrophoresis.

The NEAR™ reaction including templates for both a *Bacillus subtilis (Bs)* and *Bacillus anthracis (Ba)* DNA was run in the absence of target DNA (negative), in the presence of *Bacillus subtilis* DNA only (27mer product), and in the presence of both *Bacillus subtilis* and *Bacillus anthracis* DNA (27mer and 25mer product respectively). The target copy number for each genome present in this assay was 500,000 copies. All reactions contained 500,000 copies of *Bacillus thuringiensis* as exogenous nucleic acids. Templates varied in concentration between the assays to control the amplification. The assay was run for 10 min at 57 °C, heat denatured at 94 °C for 4 min, and 6 microliters was loaded on to a 20% gel run at 160 V for about 2 hours. The gel was stained with SYBR II fluorescent dye and imaged. The fluorescent bands were quantified and analyzed as the integrated optical density (IOD).

Figure 18. Gel electrophoresis results for the MS2/Ebola RNA duplex reaction.

The NEAR™ reaction including templates for both a MS2 and Ebola assay was run in the absence of target RNA (negative, lanes 2-5), in the presence of MS2 only (27mer product, lanes 6 and 7), and in the presence of both MS2 and Ebola RNA (27mer and 25mer product respectively, lanes 8 and 9). The target copy number used in this assay was 1E+6 copies. The assay was run for 10 min at 57 °C. Templates varied in concentration between the assays to control the amplification. Samples were run on a 20% polyacrylamide gel at 160 V for ~2.5 hours. The gel was stained with SYBR II fluorescent dye and imaged. The fluorescent bands were quantified and analyzed as the integrated optical density (IOD).

Figure 19. Mass spec analysis of amplification of DNA from lysed spores.

Average AUC values from amplified product masses compared for lysed and unlysed samples. Lysed spore samples were then added to master mix and run for 10 minutes at 55 °C, heat denatured for 4 minutes at 94 °C, and run on the mass spec for analysis. AUC values of product peaks were averaged and compared (n = 3).

Figure 20. Demonstration of the capture and extension approach for surface detection.

A.) Average binding (positive reaction product with no added polymerase), B.) 500,000 target (positive reaction product with added polymerase), and C.) No target (negative reaction with added polymerase) are compared. The NEAR™ assay was run for 10 minutes at 55 °C, heat denatured at 94 °C for 4 minutes, then added to the plate with capture probe bound to the surface on the 5' end. Polymerase is added to one well of the positive reaction. The plate is incubated at 55 °C for 30 min, washed, SYBR II added, washed 3 times, and read on a Tecan plate reader (495 nm excitation/530 nm emission).

Figure 21. Pseudo-real-time fluorescence detection of the NEAR™ FRET assay with a single template immobilized on a surface in the presence (squares) and absence (open triangles) of 1E+6 copies of genomic DNA.

Reactions were performed in flat bottom 96-well plates covered with neutravidin. A solution of 1 micromolar FRET-labeled reverse template was incubated with gentle mixing for 1 hr at 37 °C. Wells were washed 3 times with a PBS-Tween solution to release unbound template. NEAR™ reaction mix of the present method was added to the wells (one for each time point taken) and incubated at 58°C on a heating block in a shaking incubator set to 135 RPM. Time points were taken by adding 1 microliter EDTA to the well to stop the reaction. The fluorescence was read from the bottom using a Tecan 100 plate reader.

Figure 22. Limit of Detection Assay for *Chlamydia trachomatis*. A series of assays was performed using 2-fold dilutions of *Chlamydia* target. A) Bar graph of fluorescence detection showing the limit of detection as averaged from 3 assays. B) Bar graph showing the results of individual assays.

Figure 23. Discrimination of *Listeria monocytogenes* from *L. innocua*. Bar graph showing the results of a series of assays that was performed to determine the ability of the assays to discriminate between two different bacteria.

Figure 24. Assay with Viral RNA. Bar graph showing the results of a series of assays of the present methods with various dilutions of a viral RNA target.

Figure 25. Bar graph showing the results of anAssay for etection of the *bar* gene target sequence.

Figure 26. Bar graph showing the results of a assay of the present methods for detection of an miRNA target

sequence.

Figure 27. Gc Assay: LOD. A) Bar graph showing the average of a series of assays for detection of a genomic target sequence. B) Results of individual assays, including 50 genomic copies each.

Figure 28. *B. subtilis* NEAR™ assay. A) Standard curve to determine correlation between amount of reference oligonucleotide added to a sample and area under the curve (AUC). B) Bar graph showing the results of assays of the present methods to determine the amount of specific product generated. C) Table showing results of the assay.

Figure 29. Spacer length study. A) Bar graph showing the results of anassay of the present methods to determine the effect of various spacer lengths. B) Template sequences used to obtain different spacer lengths.

Figure 30. Template designs used for the assay shown in Figure 29.

Figure 31. Effect of stabilizing region. A) Graph of the results of assays of the present methods using oligo templates that either include, or don't include, stabilizing regions. B) Expansion of part of the graph of A).

Figure 32. Titration of Mg^{+2} concentration A) Bar graph showing the results of set of NEAR assays using varying amounts of Mg^{+2}. B) Chart describing components of assays.

Figure 33. Drawing depicting mechanisms of the reactions of the present methods.

Figure 34. List of examples of target and oligo template sequences.

Detailed Description

[0036] Provided herein are methods for the exponential amplification of short DNA or RNA sequences.

[0037] Target nucleic acids of the present invention include double-stranded and single-stranded nucleic acid molecules. The nucleic acid may be, for example, DNA or RNA. Where the target nucleic acid is an RNA molecule, the molecule may be, for example, double-stranded, single-stranded, or the RNA molecule may comprise a target sequence that is single-stranded. Where the target nucleic acid is an RNA molecule, the molecule may be double-stranded or single-stranded, or may comprise a target sequence that is single-stranded. Target nucleic acids include, for example, genomic, plasmid, mitochondrial, cellular, and viral nucleic acid. The target nucleic acid may be, for example, genomic, chromosomal, plasmid DNA, a gene, any type of cellular RNA, or a synthetic oligonucleotide. By "genomic nucleic acid" is meant any nucleic acid from any genome, for example, including animal, plant, insect, and bacterial genomes, including, for example, genomes present in spores. Double stranded DNA target nucleic acids include, for example, genomic DNA, plasmid DNA, mitochondrial DNA, viral DNA, and synthetic double stranded DNA or other types of DNA described herein or known in the art. Single-stranded DNA target nucleic acids include, for example, viral DNA, cDNA, and synthetic single-stranded DNA, or other types of DNA described herein or known in the art. RNA target nucleic acids include, for example, messenger RNA, viral RNA, ribosomal RNA, transfer RNA, microRNA and microRNA precursors, and siRNAs or other RNAs described herein or known in the art.

[0038] MicroRNAs, miRNAs, or small temporal RNAs (stRNAs), are short single-stranded RNA sequences, about 21-23 nucleotides long that are involved in gene regulation. MicroRNAs are thought to interfere with the translation of messenger RNAs as they are partially complementary to messenger RNAs. (see, for example, Ruvkun, G1, Science 294:797-99 (2001); Lagos-Quintana, M., et al., Science 294:854-58 (2001); Lau, N.C., et al, Science 294:858-62 (2001); Lee, R.C., and Ambros, V., Science 294:862-64 (2001); Baulcombe, D., et al., Science 297:2002-03 (2002); Llave, C., Science 297:2053-56 (2002); Hutvagner, G., and Zamore, P.D., Science 297:2056-60 (2002)). MicroRNA may also have a role in the immune system, based on studies recently reported in knockout mice. (see, for example, Wade, N., "Studies Reveal and Immune System Regulator" New York Times, April 27, 2007). MicroRNA precursors that may also be detected using the methods of the present invention include, for example, the primary transcript (primiRNA) and the pre-miRNA stem-loop-structured RNA that is further processed into miRNA.

[0039] Short interfering RNAs, or siRNAs are at least partially double-stranded, about 20-25 nucleotide long RNA molecules that are found to be involved in RNA interference, for example, in the down-regulation of viral replication or gene expression (see for example Zamore et al., 2000, Cell, 101, 25-33; Bass, 2001, Nature, 411, 428-429; Elbashir et al., 2001, Nature, 411, 494-498; and Kreutzer et al., International PCT Publication No. WO 00/44895; Zernicka-Goetz et al., International PCT Publication No. WO 01/36646; Fire, International PCT Publication No. WO 99/32619; Plaetinck et al., International PCT Publication No. WO 00/01846; Mello and Fire, International PCT Publication No. WO 01/29058; Deschamps-Depaillette, International PCT Publication No. WO 99/07409; and Li et al., International PCT Publication No. WO 00/44914; Allshire, 2002, Science, 297, 1818-1819; Volpe et al., 2002, Science, 297, 1833-1837; Jenuwein, 2002, Science, 297, 2215-2218; and Hall et al., 2002, Science, 297, 2232-2237; Hutvagner and Zamore, 2002, Science, 297, 2056-60; McManus et al., 2002, RNA, 8, 842-850; Reinhart et al., 2002, Gene & Dev., 16, 1616-1626; and Reinhart & Bartel, 2002, Science, 297, 1831).

[0040] The use of the term "target sequence" may refer to either the sense or antisense strand of the sequence, and also refers to the sequences as they exist on target nucleic acids, amplified copies, or amplification products, of the original target sequence. The amplification product may be a larger molecule that comprises the target sequence, as well as at least one other sequence, or other nucleotides. The length of the target sequence, and the guanosine:cytosine

(GC) concentration (percent), is dependent on the temperature at which the reaction is run; this temperature is dependent on the stability of the polymerases and nicking enzymes used in the reaction. Those of ordinary skill in the art may run sample assays to determine the appropriate length and GC concentration for the reaction conditions. For example, where the polymerase and nicking enzyme are stable up to 60°C, then the target sequence may be, for example, from 19 to 50 nucleotides in length, or for example, from 20 to 45, 20 to 40, 22 to 35, or 23 to 32 nucleotides in length. The GC concentration under these conditions may be, for example, less than 60%, less than 55%, less than 50%, or less than 45%. The target sequence and nicking enzymes are selected such that the target sequence does not contain nicking sites for any nicking enzymes that will be included in the reaction mix.

[0041] The target sequences may be amplified from many types of samples including, but not limited to samples containing spores, viruses, cells, nucleic acid from prokaryotes or eukaryotes, or any free nucleic acid. For example, the assay can detect the DNA on the outside of spores without the need for lysis. The sample may be isolated from any material suspected of containing the target sequence. For example, for animals, for example, mammals, such as, for example, humans, the sample may comprise blood, bone marrow, mucus, lymph, hard tissues, for example, liver, spleen, kidney, lung, or ovary, biopsies, sputum, saliva, tears, feces, or urine. Or, the target sequence may be present in air, plant, soil, or other materials suspected of containing biological organisms.

[0042] Target sequences may be present in samples that may also contain environmental and contaminants such as dust, pollen, and soot (for example, from diesel exhaust), or clinically relevant matrices such as urine, mucus, or saliva. Target sequences may also be present in waste water, drinking water, air, milk, or other food. Depending on the concentration of these contaminants, sample purification methods known to those of ordinary skill in the art may be required to remove inhibitors for successful amplification. Purification may, for example, involve the use of detergent lysates, sonication, vortexing with glass beads, or a French press. This purification could also result in concentration of the sample target. Samples may also, for be further purified, for example, by filtration, phenol extraction, chromatography, ion exchange, gel electrophoresis, or density dependent centrifugation. In particular embodiments, the sample can be added directly to the reaction mix or pre-diluted and then added to the reaction mix without prior purification of target nucleic acid.

[0043] An oligonucleotide is a molecule comprising two or more deoxyribonucleotides or ribonucleotides, for example, more than three. The length of an oligonucleotide will depend on how it is to be used. The oligonucleotide may be derived synthetically or by cloning.

[0044] The term "complementary" as it refers to two nucleic acid sequences generally refers to the ability of the two sequences to form sufficient hydrogen bonding between the two nucleic acids to stabilize a double-stranded nucleotide sequence formed by hybridization of the two nucleic acids. In the two sequences, all nucleotides in one sequence may be complementary to counterpart nucleotides in the other sequence. In some embodiments, there may be a few mismatches between counterpart nucleotides in the two sequences (i.e., non-complementary nucleotides), such as 1 mismatch in 10 nucleotides, 1 mismatch in 20 nucleotides, or 1 mismatch in 30 nucleotides, for example, which sequences are referred to as "substantially complementary" herein. As shown in Figures 1A-1D, each template nucleic acid often includes a recognition region complementary to, or substantially complementary to, a target nucleic acid strand (or complement thereof) to which the template nucleic acid hybridizes. Also shown in Figures 1A-1D, each template nucleic acid often includes a stabilizing region 5' of the recognition region and nick agent recognition region that is not complementary or substantially complementary to the target nucleic acid sequence or complement thereof.

[0045] As used herein, "hybridization" and "binding" are used interchangeably and refer to the non-covalent binding or "base pairing" of complementary nucleic acid sequences to one another. Whether or not a particular probe remains base paired with a polynucleotide sequence depends on the degree of complementarity, the length of the probe, and the stringency of the binding conditions. The higher the stringency, the higher must be the degree of complementarity, and/or the longer the probe for binding or base pairing to remain stable.

[0046] As used herein, "stringency" refers to the combination of conditions to which nucleic acids are subjected that cause double-stranded nucleic acid to dissociate into component single strands such as pH extremes, high temperature, and salt concentration. The phrase "high stringency" refers to hybridization conditions that are sufficiently stringent or restrictive such that only specific base pairings will occur. The specificity should be sufficient to allow for the detection of unique sequences using an oligonucleotide probe or closely related sequence under standard Southern hybridization protocols (as described in J. Mol. Biol. 98:503 (1975)).

[0047] Templates are defined as oligonucleotides that bind to a recognition region of a target sequence and also contain a nicking enzyme binding region upstream of the recognition region and a stabilizing region upstream to the nicking enzyme binding region.

[0048] By "recognition region" is meant a nucleic acid sequence on the template that is complementary or substantially complementary to a nucleic acid sequence on the target sequence. By "recognition region on the target sequence" is meant the nucleotide sequence on the target sequence that is complementary or substantially complementary to, and binds to, the template.

[0049] By "stabilizing region" is meant a nucleic acid sequence having, for example, about 50% GC content, designed

to stabilize the molecule for, for example, the nicking and/or extension reactions.

[0050] In describing the positioning of certain sequences on nucleic acid molecules, such as, for example, in the target sequence, or the template, it is understood by those of ordinary skill in the art that the terms "3' " and "5' " refer to a location of a particular sequence or region in relation to another. Thus, when a sequence or a region is 3' to or 3' of another sequence or region, the location is between that sequence or region and the 3' hydroxyl of that strand of nucleic acid. When a location in a nucleic acid is 5' to or 5' of another sequence or region, that means that the location is between that sequence or region and the 5' phosphate of that strand of nucleic acid.

[0051] The polymerase is a protein able to catalyze the specific incorporation of nucleotides to extend a 3' hydroxyl terminus of a primer molecule, such as, for example, the template oligonucleotide, against a nucleic acid target sequence. The polymerase may be, for example, thermophilic so that it is active at an elevated reaction temperature. It may also, for example, have strand displacement capabilities. It does not, however, need to be very processive (30-40 nucleotides for a single synthesis are sufficient). Often, the polymerase used does not have 5'-3' exonuclease activity. If the polymerase also has reverse transcriptase activity (such as Bst (large fragment), 9°N, Therminator, Therminator II, etc.) the reaction can also amplify RNA targets in a single step without the use of a separate reverse transcriptase. More than one polymerase may be included in the reaction, in one example one of the polymerases may have reverse transcriptase activity and the other polymerase may lack reverse transcriptase activity. In exemplary embodiments, the polymerase is BST (large fragment). The polymerase may be selected from, for example, the group consisting of one or more of the polymerases listed in Table 1.

Table 1

Polymerase
Bst DNA polymerase
Bst DNA polymerase (Large fragment)
9°Nm DNA polymerase
Phi29 DNA polymerase
DNA polymerase I (*E.coli*)
DNA polymerase I, Large (Klenow) fragment
Klenow fragment (3'-5' exo-)
T4 DNA polymerase
T7 DNA polymerase
Deep Vent$_R$™ (exo-) DNA Polymerase
Deep Vent$_R$™ DNA Polymerase
DyNAzyme™ EXT DNA
DyNAzyme™ II Hot Start DNA Polymerase
Phusion™ High-Fidelity DNA Polymerase
Therminator™ DNA Polymerase
Therminator™ II DNA Polymerase
Vent$_R$® DNA Polymerase
Vent$_R$® (exo-) DNA Polymerase
RepliPHI™ Phi29 DNA Polymerase
rBst DNA Polymerase
rBst DNA Polymerase, Large Fragment (IsoTherm™ DNA Polymerase)
MasterAmp™ AmpliTherm™ DNA Polymerase
Taq DNA polymerase
Tth DNA polymerase
Tfl DNA polymerase

(continued)

Polymerase
Tgo DNA polymerase
SP6 DNA polymerase
Tbr DNA polymerase
DNA polymerase Beta
ThermoPhi DNA polymerase
Pyrophage 3173 (Lucigen)

[0052] The following non-limiting examples of Reverse Transcriptases (RT) can be used in the reactions of the present method to improve performance when detecting an RNA sequence: OmniScript (Qiagen), SensiScript (Qiagen), MonsterScript (Epicentre), Transcriptor (Roche), HIV RT (Ambion), SuperScript III (Invitrogen), ThermoScript (Invitrogen), Thermo-X (Invitrogen), ImProm II (Promega).

[0053] These different RTs perform at different levels in the standard reaction buffer, and this performance rating is listed below. A "+" indicates that the amplification reaction results in specific product. More "+"s indicate that the reaction works better, with "+++++" indicating excellent results. A "-" indicates that the reaction did not result in specific product, or did not result in specific product over background.

Table 2

OmniScript** (Qiagen)	+++++
SensiScript (Qiagen)	+++
MonsterScript (Epicentre)	+++
Transcriptor (Roche)	++
HIV RT* (Ambion)	+
SuperScript III (Invitrogen)	-
ThermoScript (Invitrogen)	-
Thermo-X (Invitrogen)	-
ImProm II (Promega)	-

[0054] "Nicking" refers to the cleavage of only one strand of the double-stranded portion of a fully or partially double-stranded nucleic acid. The position where the nucleic acid is nicked is referred to as the nicking site or nicking site. The recognition sequence that the nicking enzyme recognizes is referred to as the nicking enzyme binding site. "Capable of nicking" refers to an enzymatic capability of a nicking enzyme.

[0055] The nicking enzyme is a protein that binds to double-stranded DNA and cleaves one strand of a double-stranded duplex. The nicking enzyme may cleave either upstream or downstream of the binding site, or nicking enzyme recognition site. In exemplary embodiments, the reaction comprises the use of nicking enzymes that cleave or nick downstream of the binding site (top strand nicking enzymes) so that the product sequence does not contain the nicking site. Using an enzyme that cleaves downstream of the binding site allows the polymerase to more easily extend without having to displace the nicking enzyme. The nicking enzyme must be functional in the same reaction conditions as the polymerase, so optimization between the two ideal conditions for both is necessary. Nicking enzymes are available from, for example, New England Biolabs (NEB) and Fermentas. The nicking enzyme may, for example, be selected from the group consisting of one or more of the nicking enzymes listed in Table 3.

Table 3

Nicking Enzyme	Alternate Name
Nb.BbvCI	
Nb.Bpu10I	
Nb.BsaI	

(continued)

Nicking Enzyme	Alternate Name
Nb.BsmI	
Nb.BsrDI	
Nb.BstNBIP	
Nb.BstSEIP	
Nb.BtsI	
Nb.SapI	
Nt.A1wI	
Nt.BbvCI	
Nt.BhaIIIP	
Nt.Bpu10I	
Nt.Bpu10IB	
Nt.BsaI	
Nt.BsmAI	
Nt.BsmBI	
Nt.BspD6I	
Nt.BspQI	
Nt.Bst9I	
Nt.BstNBI	N.BstNB I
Nt.BstSEI	
Nt.CviARORFMP	
Nt.CviFRORFAP	
Nt.CviPII	Nt.CviPIIm
Nt.CviQII	
Nt.CviQXI	
Nt.EsaSS1198P	
Nt.MlyI	
Nt.SapI	

[0056] Nicking enzymes may be, for example, selected from the group consisting of Nt.BspQI(NEB), Nb.BbvCI(NEB), Nb.BsmI(NEB), Nb.BsrDI(NEB), Nb.BtsI(NEB), Nt.A1wI(NEB), Nt.BbvCI(NEB), Nt.BstNBI(NEB), Nt.CviPII(NEB), Nb.Bpu101(Fermantas), and Nt.Bpu101(Fermentas). In certain embodiments, the nicking enzyme is selected from the group consisting of Nt.NBst.NBI, Nb.BsmI, and Nb.BsrDI. Those of ordinary skill in the art are aware that various nicking enzymes other than those mentioned specifically herein may be used in the present methods.

[0057] Nicking enzymes and polymerases of the present methods may be, for example, stable at room temperature, the enzymes may also, for example, be stable at temperatures up to 37°C , 42°C , 60°C, 65°C, 70°C, 75°C, 80°C, or 85°C. In certain embodiments, the enzymes are stable up to 60°C.

[0058] An enzyme is "thermophilic" when it is stable at temperatures up to 37°C , 42°C , 50-60C, 54-60°C, 56-58°C, 60°C, 65°C, 70°C, 75°C, 80°C, or 85°C

[0059] Product or amplified product is defined as the end result of the extension of the template along the target that is nicked and released. This product can then feed back into the amplification cycle, or it can anneal to its complement or a molecular beacon.

[0060] A "native nucleotide" refers to adenylic acid, guanylic acid, cytidylic acid, thymidylic acid, or uridylic acid. A "derivatized nucleotide" is a nucleotide other than a native nucleotide.

[0061] The reaction may be conducted in the presence of native nucleotides, such as, for example, dideoxyribonucleoside triphosphates (dNTPs). The reaction may also be carried out in the presence of labeled dNTPs, such as, for example, radiolabels such as, for example, ^{32}P, ^{33}P, ^{125}I, or ^{35}S, enzyme labels such as alkaline phosphatase, fluorescent labels such as fluorescein isothiocyanate (FITC), biotin, avidin, digoxigenin, antigens, haptens, or fluorochromes. These derivatized nucleotides may, optionally, be present in the templates.

[0062] By "constant temperature," "isothermal conditions," "essentially isothermal," or "isothermally" is meant a set of reaction conditions where the temperature of the reaction is kept essentially or substantially constant during the course of the amplification reaction. An advantage of the amplification method of the present methods is that the temperature does not need to be cycled between an upper temperature and a lower temperature. The nicking and the extension reaction will work at the same temperature or within the same narrow temperature range. However, it is not necessary that the temperature be maintained at precisely one temperature. If the equipment used to maintain an elevated temperature allows the temperature of the reaction mixture to vary by a few degrees, or few tenths of a degree, such as, for example, less than 1 degree, 0.8 degrees, 0.6 degrees, 0.4 degrees, or 0.2 degrees, this is not detrimental to the amplification reaction, and may still be considered to be an isothermal reaction.

[0063] The term "multiplex amplification" refers to the amplification of more than one nucleic acid of interest. For example, it can refer to the amplification of multiple sequences from the same sample or the amplification of one of several sequences in a sample as discussed, for example, in U.S. Patent Nos. 5,422,252; and 5,470,723, which provide examples of multiplex strand displacement amplification. The term also refers to the amplification of one or more sequences present in multiple samples either simultaneously or in step-wise fashion.

Template Design

[0064] Forward and Reverse templates, and first and second templates, are designed so that there is a stabilizing region at the 5' end, a nicking enzyme binding site and a nicking site downstream of the stabilizing region, and a recognition region downstream of the nicking enzyme binding site and the nicking site on the 3' end of the oligonucleotide. The total oligo length can range from 19 to 40, for example from 19-40, 23-40, 20-30, 20-24, 23-24, 23-32, 25-40, 27-40, or 27-35 nucleotides depending on the length of each individual region, the temperature, the length of the target sequence, and the GC concentration. One of ordinary skill in the art would know how to balance these features of the templates. The templates may be designed so that they, together, would bind to less than or equal to 100% of the target sequence, one binding to the sense strand, and one to the antisense strand. The length of each template does not need to be the same length as the other template. For example, where the forward template binds to about 60% of the target antisense strand, the reverse template may, for example, bind to about 40% of the target sense strand. The templates may be designed to allow for spacer bases on the target sequence, that do not bind to either template. The templates thus may be designed to bind to about 30%, about 40%, about 50%, or about 60% of the target sequence.

[0065] The recognition region of the forward template is designed to be substantially identical or identical to the 5' region of the target sense strand and complementary or substantially complementary to the 3' end of the target site antisense strand. The recognition region of the forward template is of any suitable length, for example, about 8, 9, 10, 11, 12, 13, 14, 15 or 16 bases in length, and sometimes 8-16, 9-16, 10-16, 8-12, 8-15, 9-15, 10-15, or 11-14 nucleotides long. In exemplary embodiments, the length is 11-13, 11-12, 12, or 12-13 nucleotides long. The recognition region of the reverse template is designed to be substantially complementary or complementary to the 3' end of the target site sense strand. The recognition region of the reverse template is of any suitable length, for example, about 8, 9, 10, 11, 12, 13, 14, 15 or 16 bases in length, and sometimes 8-16, 9-16, 10-16, 8-12, 8-15, 9-15, 10-15, or 11-14 nucleotides long. In exemplary embodiments, the length is 11-13, 11-12, 12, or 12-13 nucleotides long. The length of the recognition region of the first template may either be the same as the length of the recognition region of the second template, or may be different.

[0066] A recognition sequence of a template often is complementary or substantially complementary to a unique sequence, or substantially unique sequence, of an organism. The term "unique sequence" as used herein refers to a nucleotide sequence in an organism that is present in no other known organism. A "substantially unique sequence" as used herein refers to a nucleotide sequence present in a specific family of organisms, or in up to only about 1, 2, 3, 4, 5, 6, 7, 8, 9, or 10 other organisms. In some embodiments, a unique sequence or substantially unique sequence is present in ribosomal RNA or in the sense or antisense strand of DNA encoding ribosomal RNA.

[0067] Those of ordinary skill in the art are able to determine the appropriate recognition region length for optimal, efficient, amplification. In certain embodiments, to provide appropriate specificity, an 8 base-length template recognition region is a lower limit. The analytical specificity of the reaction is linked to the sum of the recognition regions of the two templates, the forward and the reverse template. If each template has a recognition region of 8 nucleotides, for example, that confers an assay that is able to detect a unique combination of 8+8=16 nucleotides, referred to as the "target size." For a given DNA strand, a target size of 16 nucleotides has 4.29x109 possible combinations. The human genome is 3.3x109 nucleotides long. Therefore, statistically, a specific 16 nucleotide sequence is expected to occur approximately

once in the human genome. As the target size decreases, for example to 15 nucleotides, that would be expected to occur, on average, 3 times in the human genome (1.07x109 possibilities in 3.3x109 occurrences), and would therefore not be as specific as a 16 nucleotide target size. For an assay with a recognition region of 7 nucleotides, conferring an assay target size of 14 bases, this would be expected to be present in the human genome 12 times (2.68x108 possibilities in 3.3x109 occurrences). This would generate an assay with reduced specificity that would have less value in a diagnostic setting. Therefore, an 8 base recognition region for each template is often considered to be the lower limit for certain assays.

Table 4

Assay Target Size	# unique possibilities
N	4^N
14	2.68E+08
15	1.07E+09
16	4.29E+09
17	1.72E+10
18	6.87E+10
19	2.75E+11
20	1.10E+12
21	4.40E+12
22	1.76E+13
23	7.04E+13
24	2.81E+14
25	1.13E+15
26	4.50E+15

[0068] Amplification ssays in accordance with the present invention were conducted to determine the optimal length of the recognition region. In 10 minute assays, using either 0 or 100,000 copies of target DNA, a 20 mer recognition region template set did not produce detectable specific product, while specific product was detected using a 12 mer recognition region template set. The use of a 16 mer recognition region template set resulted in specific detectable product, but four-fold less specific product was detected than in an assay using the 12 mer recognition region template set. In certain embodiments, the use of a 15 mer recognition region template set generated more specific product than a 16 mer recognition region template set.

[0069] Thus, in certain exemplary embodiments, methods are provided for amplifying a double stranded nucleic acid target sequence comprising contacting a target DNA molecule comprising a double-stranded target sequence, having a sense strand and an antisense strand, with a forward template and a reverse template, wherein the forward template comprises a nucleic acid sequence comprising a recognition region at the 3' end that is complementary or substantially complementary to the 3' end of the target sequence antisense strand, wherein the recognition region is from 8 to 15 nucleotides in length; a nicking enzyme binding site and a nicking site upstream of the recognition region and a stabilizing region upstream of the nicking enzyme binding site and the nicking site; the reverse template comprises a nucleotide sequence comprising a recognition region at the 3' end that is complementary or substantially complementary to the 3' end of the target sequence sense strand, wherein the recognition region is from 8 to 15 nucleotides in length, a nicking enzyme binding site and a nicking site upstream of the recognition region, and a stabilizing region upstream of the nicking enzyme binding site and the nicking site; providing a first nicking enzyme that is capable of nicking at the nicking site of the forward template, and does not nick within the target sequence; providing a second nicking enzyme that is capable of nicking at the nicking site of the reverse template and does not nick within the target sequence; and providing a DNA polymerase; under conditions wherein amplification is performed by multiple cycles of the polymerase extending the forward and reverse templates along the target sequence producing a double-stranded nicking site, and the nicking enzymes nicking at the nicking sites, producing an amplification product. Thus, in certain embodiments, the recognition region of the forward or reverse template, or each of the forward and reverse templates is 8, 9, 10, 11, 12, 13, 14, or 15 nucleotides in length. In certain embodiments, the target sequence comprises from 1 to 5 nucleotides more than the sum of the nucleotides of the forward template recognition region and the reverse template recognition region

[0070] In another exemplary embodiment, methods are provided for amplifying a single-stranded nucleic acid target sequence, comprising contacting a target nucleic acid comprising a single-stranded target sequence with a reverse template, wherein the reverse template comprises a nucleotide sequence comprising a recognition region at the 3' end that is complementary or substantially complementary to the 3' end of the target sequence wherein the recognition

region is from 8 to 15 nucleotides in length, a nicking enzyme binding site and a nicking site upstream of the recognition region, and a stabilizing region upstream of the nicking enzyme binding site and the nicking site; providing a first nicking enzyme that is capable of nicking at the nicking site of the reverse template, and does not nick within the target sequence; providing a DNA polymerase under conditions wherein the polymerase extends the reverse template along the target sequence; contacting the extended reverse template with a forward template, wherein the forward template comprises a recognition region at the 3'end that is identical to the 5'end of the target sequence wherein the recognition region is from 8 to 15 nucleotides in length, a nicking enzyme binding site and a nicking site upstream of the recognition region, and a stabilizing region upstream of the nicking enzyme binding site and the nicking site; providing a second nicking enzyme that is capable of nicking at the nicking site of the forward template and does not nick within the target sequence; under conditions wherein amplification is performed by multiple cycles of the polymerase extending the forward and reverse templates along the target sequence producing a double-stranded nicking site, and the nicking enzymes nicking at the nicking sites, producing an amplification. Thus, in certain embodiments, the recognition region of the forward or reverse template, or each of the forward and reverse templates, is 8, 9, 10, 11, 12, 13, 14, or 15 nucleotides in length. In certain embodiments, the target sequence comprises from 1 to 5 nucleotides more than the sum of the nucleotides of the forward template recognition region and the reverse template recognition region

[0071] In certain embodiments, the temperature at which the amplification reaction is conducted is lower than the melting temperature (T_m) of a template and target. In certain embodiments, the reaction temperature is, for example, from 1 °C -10 °C, 1 °C -8 °C, 1 °C - 6 °C, 1 °C -4 °C, 1 °C -2 °C, 2 °C -10 °C, 2 °C -8 °C, 2 °C -6 °C, 2 °C -4 °C, 2 °C -2 °C from 2 °C-4 °C or from 2 °C, 3 °C, 4 °C, 5 °C, 6°C, 7°C, or 8°C less than the T_m of a template and target. The reaction temperature also often is lower than the T_m of the reaction products (e.g., products of nicking and polymerase extension of the amplification duplex shown in Figure 1B and Figure 1C after step 9A). The reaction temperature may be higher than the T_m of the initial template/target sequence complex (drawing above step (1) of Figure 1A). Once the template is extended to form a stable complex, the T_m of the stable complex is higher than the reaction temperature.

[0072] Thus, the T_m of a template/target nucleic acid target often is higher than the reaction temperature, and sometimes the T_m is 5 °C or more higher than the reaction temperature, or for example, about 1 °C, 2 °C, 3 °C, 4 °C, 5 °C, 6 °C, 7 °C, or 8 °C or more higher than the reaction temperature. The T_m of each portion of the nicked strand after nicking often is higher than the reaction temperature, and sometimes the T_m of each nicked portion is 5 °C or more higher than the reaction temperature, or for example, about 1 °C, 2 °C, 3 °C, 4 °C, 5 °C, 6 °C, 7 °C, or 8 °C or more higher than the reaction temperature. The T_m of the template and target may be calculated, for example, using the program provided for the IDT Oligo Analyzer (Integrated DNA Technologies) at World Wide Web URL idtdna.com/analyzer/Applications/OligoAnalyzer/ considering the salt concentration of the reaction conditions. As discussed at the IDT website, the T_m calculations using the Analyzer are conducted as follows:

[0073] Melting temperature (T_M) is the temperature at which an oligonucleotide duplex is 50% in single-stranded form and 50% in double-stranded form. The Oligo Analyzer estimates T_M from the nearest-neighbor two-state model, which is applicable to short DNA duplexes,

$$T_M(^\circ C) = \frac{\Delta H^\circ}{\Delta S^\circ + R \ln[\text{oligo}]} - 273.15$$

where ΔH° (enthalpy) and ΔS° (entropy) are the melting parameters calculated from the sequence and the published nearest neighbor thermodynamic parameters, R is the ideal gas constant (1.987 $cal \cdot K^{-1} mole^{-1}$), [oligo] is the molar concentration of an oligonucleotide, and the constant of -273.15 converts temperature from Kelvin to degrees of Celsius. The most accurate, nearest-neighbor parameters were obtained from the following publications for DNA/DNA base pairs (Allawi,H., SantaLucia,J.,Jr., Biochemistry, 36, 10581), RNA/DNA base pairs (Sugimoto,N. et al., Biochemistry, 34, 11211), RNA/RNA base pairs (Xia,T. et al., Biochemistry, 37, 14719), and LNA/DNA base pairs (McTigue,P.M. et al., Biochemistry, 43, 5388).

[0074] T_M depends on monovalent salt concentration ([Na^+]) of the solvent. The linear T_M correction is a method known in the art. As discussed in the IDT website, scientists at IDT performed a large set of UV melting experiments (~3000 measurements) on about 100 short DNA duplexes in a variety of sodium buffers and determined that this linear function is inaccurate. OligoAnalyzer employs the improved quadratic T_M salt correction function (Owczarzy,R. et al., Biochemistry, 43, 3537),

$$\frac{1}{T_M(Na^+)} = \frac{1}{T_M(1M\ Na^+)} + \left(4.29 f(GC) - 3.95\right) \times 10^{-5} \ln[Na^+] + 9.40 \times 10^{-6} \ln^2[Na^+]$$

where f(GC) is the fraction of GC base pairs.

[0075] In certain embodiments, the lengths of the recognition regions are adjusted so that there is at least one nucleotide in the target sequence that is not in the forward template's recognition region and also does not have its complement in the reverse template's recognition region. These spacer bases (which form the "spacer region") are nucleotides contained within the target sequence that lie in between the 3' ends of the forward and reverse templates. The spacer bases are shown in, for example, Figure 30, where they are indicated as the section of the target sense and antisense sequences between the 3' ends of the forward and reverse templates, also indicated within the "spacer region." For example, when templates T2 and T1 of Figure 30 are used with the target, the target sense strand has 1 spacer base (or, a gap of 1 nucleotide)-T, and the target antisense strand has 1 spacer base (or, a gap of 1-nucleotide)-A. In certain embodiments, 5 spacer bases or less are present in the target sequence. In exemplary embodiments, the number of spacer bases is 2 to 3. In certain embodiments, the number of spacer bases is 1, 2, or 3. In other embodiments, there is 1 spacer base. In other embodiments, there are 2 spacer bases. In other embodiments, there are 3 spacer bases. In other embodiments, the number of spacer bases is 1, 2, 3, 4, or 5.

[0076] Thus, in exemplary embodiments of the present methods, the target sequence comprises from 1 to 5 nucleotides between the target sequence nucleotide that hybridizes to the 3' end of the first template and the corresponding nucleotide to the nucleotide of the complement of the first strand that hybridizes to the 3' end of the second template. By "corresponding nucleotide" is meant the nucleotide on one strand of the target nucleotide sequence that hybridizes to the complementary strand of the target nucleotide sequence when the two strands are aligned. These 1 to 5 nucleotides are also called spacer bases.

[0077] These spacer bases allow for distinction of the true amplified product from any background products amplified by extension due to overlapping templates in a similar manner to primer-dimers. This consideration allows for improved discrimination between background noise and amplification of a target sequence. However, these spacer bases are not required for the amplification to proceed.

[0078] Thus, in certain exemplary embodiments, methods are provided for amplifying a double stranded nucleic acid target sequence comprising contacting a target DNA molecule comprising a double-stranded target sequence, having a sense strand and an antisense strand, with a forward template and a reverse template, wherein the forward template comprises a nucleic acid sequence comprising a recognition region at the 3' end that is complementary or substantially complementary to the 3' end of the target sequence antisense strand; a nicking enzyme binding site and a nicking enzyme binding site and a nicking site upstream of the recognition region and a stabilizing region upstream of the nicking enzyme binding site and the nicking site; the reverse template comprises a nucleotide sequence comprising a recognition region at the 3' end that is complementary or substantially complementary to the 3' end of the target sequence sense strand, a nicking enzyme binding site and a nicking site upstream of the recognition region, and a stabilizing region upstream of the nicking enzyme binding site and the nicking site; providing a first nicking enzyme that is capable of nicking at the nicking site of the forward template, and does not nick within the target sequence; providing a second nicking enzyme that is capable of nicking at the nicking site of the reverse template and does not nick within the target sequence; and providing a DNA polymerase; under conditions wherein amplification is performed by multiple cycles of the polymerase extending the forward and reverse templates along the target sequence producing a double-stranded nicking site, and the nicking enzymes nicking at the nicking sites, producing an amplification product, wherein the target sequence comprises from 1 to 5 nucleotides more than the sum of the nucleotides of the forward template recognition region and the reverse template recognition region. Thus, in certain embodiments, the target sequence comprises 1, 2, 3, 4, or 5 nucleotides more than the sum of the nucleotides of the forward template recognition region and the reverse template recognition region.

[0079] In another exemplary embodiment, methods are provided for amplifying a single-stranded nucleic acid target sequence, comprising contacting a target nucleic acid comprising a single-stranded target sequence with a reverse template, wherein the reverse template comprises a nucleotide sequence comprising a recognition region at the 3' end that is complementary or substantially complementary to the 3' end of the target sequence, a nicking enzyme binding site and a nicking site upstream of the recognition region, and a stabilizing region upstream of the nicking enzyme binding site and the nicking site; providing a first nicking enzyme that is capable of nicking at the nicking site of the reverse template, and does not nick within the target sequence; providing a DNA polymerase under conditions wherein the polymerase extends the reverse template along the target sequence; contacting the extended reverse template with a forward template, wherein the forward template comprises a recognition region at the 3'end that is identical to the 5'end of the target sequence a nicking enzyme binding site and a nicking site upstream of the recognition region, and a stabilizing region upstream of the nicking enzyme binding site and the nicking site; providing a second nicking enzyme that is capable of nicking at the nicking site of the forward template and does not nick within the target sequence; under conditions wherein amplification is performed by multiple cycles of the polymerase extending the forward and reverse templates along the target sequence producing a double-stranded nicking site, and the nicking enzymes nicking at the nicking sites, producing an amplification product , wherein the target sequence comprises from 1 to 5 nucleotides more than the sum of the nucleotides of the forward template recognition region and the reverse template recognition region.

Thus, in certain embodiments, the target sequence comprises 1, 2, 3, 4, or 5 nucleotides more than the sum of the nucleotides of the forward template recognition region and the reverse template recognition region.

[0080] The nicking enzyme binding site sequence of the template depends on which nicking enzyme is chosen for each template. Different nicking enzymes may be used in a single assay, but a simple amplification may, for example, employ a single nicking enzyme for use with both templates. Thus, the embodiments of the present methods include those where both templates comprise recognition sites for the same nicking enzyme, and only one nicking enzyme is used in the reaction. In these embodiments, both the first and second nicking enzymes are the same. The present methodalso includes those embodiments where each template comprises a recognition site for a different nicking enzyme, and two nicking enzymes are used in the reaction.

[0081] For example, in the case of Nt.BstNBI, the enzyme binding site is 5'-GAGTC-3' and the enzyme nicks the top strand four nucleotides down stream of this site (i.e., GAGTCNNNN^). The amplification reaction shows little dependence on the sequence of these four nucleotides (N), though optimal sequence of this region is 25% or less GC content and with a thymine adjacent to the 5' nucleotide of the binding region. The latter stipulation allows for the priming ability of products that have an additional adenine added on by the polymerase. The sequence of the four nucleotides can be optimized to create or eliminate the presence of hairpins, self-dimers, or heterodimers, depending on the application.

[0082] The stabilizing region on the 5' end of the template oligonucleotide is designed to be roughly 50% GC. Thus, the GC content may be, for example, about 40%-60%, about 42%-58%, about 44%-56%, about 46%-54%, about 48%-52%, or about 49%-51%. These parameters result in a stabilizing region length of 8-11 nucleotides for the Nt.BstNBI enzyme, though lengths as short as 6 and as long as 15 nucleotides have been tested and were shown to work in this amplification method. Longer stabilizing regions or increased %GC to greater than 50 % could further stabilize the nicking and extension reactions at higher reaction temperatures. The sequence of the 5' stabilizing regions of forward and reverse templates are usually identical, but can be varied if the aim is to capture each product strand independently. The sequence of this region should not interfere with the nicking site or the recognition region, though short internal hairpins within the template sequence have been shown to have improved real-time results.

[0083] In certain embodiments, one or more agents that destabilize nucleic acid interaction (e.g., inter-strand or intra-strand interactions) are included in an amplification process, and in alternative embodiments, one or more of such agents are not included in an amplification process. Examples of agents that destabilize nucleic acid interaction are those that destabilize double-stranded structure (e.g., double-stranded DNA), and/or structures within a strand (e.g., secondary or tertiary structures in RNA), and include, without limitation, betaines and other tetra-ammonium compounds, formamide, glycerol, sorbitol, sodium perchlorate, dimethylsulfoxide (DMSO), lower alkyl alcohols (e.g., ethanol; 1-4 carbon alcohols), urea, trialkyl ammonium salts (e.g., triethyl ammonium chloride), single strand binding (ssb) proteins, such as, for example, E. coli ssb, helicases, such as, for example, E. coli DNA helicases I, II, or IV, lower alkyl (1-4 C) alcohols, and the like. Without being bound by theory, such agents lower the melting temperature (T_m) of nucleic acid interactions (e.g., lower duplex T_m). Those of ordinary skill in the art may determine the appropriate destabilizing agent and appropriate destabilizing agent concentration for the reaction, considering, for example, the amount of destabilization as well as the need to maintain enzymatic activity. Examples of concentrations include about 10% glycerol, about 10% sodium perchlorate, about 10% DMSO, about 10% sorbitol, about 2.4 molar triethyl ammonium chloride, and about greater than 1, 2, 3, 4, or 5 molar betaine, for example, about 5-6 molar betaine. Betaine, or N,N,N-trimethylglycine, may be purchased from, for example Sigma-Aldrich, for example, catalog numbers B2629 or B0300. It may be used, for example, in combination with low concentrations of DMSO, for example, about 1-2, or about 1.3% DMSO to about 1M betaine.

[0084] The templates of the present methods may include, for example, spacers, blocking groups, and modified nucleotides. Modified nucleotides are nucleotides or nucleotide triphosphates that differ in composition and/or structure from natural nucleotide and nucleotide triphosphates. Modified nucleotide or nucleotide triphosphates used herein may, for example, be modified in such a way that, when the modifications are present on one strand of a double-stranded nucleic acid where there is a restriction endonuclease recognition site, the modified nucleotide or nucleotide triphosphates protect the modified strand against cleavage by restriction enzymes. Thus, the presence of the modified nucleotides or nucleotide triphosphates encourages the nicking rather than the cleavage of the double-stranded nucleic acid. Blocking groups are chemical moieties that can be added to the template to inhibit target sequence-independent nucleic acid polymerization by the polymerase. Blocking groups are usually located at the 3' end of the template. Examples of blocking groups include, for example, alkyl groups, non-nucleotide linkers, phosphorothioate, alkane-diol residues, peptide nucleic acid, and nucleotide derivatives lacking a 3'-OH, including, for example, cordycepin. Examples of spacers, include, for example, C3 spacers. Spacers may be used, for example, within the template, and also, for example, at the 5' end, to attach other groups, such as, for example, labels.

[0085] Unmodified nucleotides often are provided for template extension. Unmodified nucleotides and nucleotide derivatives often are not provided for incorporation into extended templates. In certain embodiments, however, one or more modified nucleotides or nucleotide derivatives may be provided and incorporated into an extended template.

[0086] The amplification reaction may also include helper oligonucleotides. Helper oligonucleotides are oligonucleotides that are, for example, about 5-10, 5-15, 5-20, nucleotides long. The presence of helper oligonucleotides may

increase the speed, amount, or sensitivity of the amplification reaction. Helper oligonucleotides are not incorporated into the final product. Those of ordinary skill in the art would be able to determine the appropriate helper oligonucleotides to add to a reaction, as well as the amount to add. One example of a way to determine the appropriate helper oligonucleotides is to synthesize oligonucleotides that are complementary to various regions of the target nucleic acid or its complement, and add them to the assay in varying amounts, comparing the assay with the helper oligonucleotides to one without helper oligonucleotides as a control. Helper oligonucleotides may be synthesized that are complementary to regions upstream or downstream of the recognition region, or its complement. For example, sets of helper oligonucleotides about 10 bases long may be synthesized that are complementary to regions spaced every 5-10 bases upstream or downstream of the recognition region, then tested in pairs for their ability to enhance the amplification reaction.

Detailed Mechanism of Amplification

[0087] Amplification reactions of the present methods require the presence of a nucleic acid target, at least two template oligonucleotides, a nicking enzyme, for example, a thermophilic nicking enzyme, a thermophilic polymerase, and buffer components all held at the reaction temperature. The recognition region of the templates interacts with the complementary or substantially complementary target sequence. Since the melting temperature of the complementary or substantially complementary regions of the target and template is well below the reaction temperature, the interaction between the two nucleic acid strands is transient, but allows enough time for a thermophilic polymerase to extend from the 3' end of the template along the target strand. Experiments have shown that certain polymerases bind to single-stranded oligonucleotides. The pre-formation of this complex can facilitate the speed of the amplification process.

[0088] For a double-stranded target, both templates can interact with the corresponding target strands simultaneously (forward template with the antisense strand and reverse template with the sense strand) during the normal breathing of double-stranded DNA. The target may also be generated by a single or double nick sites within the genome sequence. For a single-stranded target (either RNA or DNA), the reverse template binds and extends first (Figure 1, Step 1 and 2). The extended sequence contains the complement to the forward template. The forward template then displaces a region of the target and binds to the 3' synthesized region complementary or substantially complementary to the recognition region of the forward template (Step 3). Alternatively, another reverse template can also displace the initial extended reverse template at the recognition region to create a single-stranded extended reverse template for the forward template to bind. The initial binding and extension of the templates is facilitated by a non-processive polymerase that extends shorter strands of DNA so that the melting temperature of the synthesized product is above the reaction temperature. The single-stranded product is then available for the next template recognition site to bind and polymerase to extend.

[0089] The forward template is extended to the 5' end of the reverse template, creating a double-stranded nicking enzyme binding site for the reverse template (Step 5). The nicking enzyme then binds to the duplex and nicks directly upstream of the recognition sequence of the reverse template strand (in the case of a top-strand nicking enzyme) (Step 6). The nucleic acid sequence downstream of the nick is either released (if the melting temperature is near the reaction temperature) and/or is displaced by the polymerase synthesis from the 3'-OH nick site. Polymerase extends along the forward template to the 5' end of the forward template (Step 8). The double-strand formed from the extension of both templates creates a nicking enzyme binding site on either end of the duplex. This double-strand is termed the NEAR™ amplification duplex. When nicking enzyme binds and nicks, either the target product located in between the two nick sites (with 5'-phosphate and 3'-OH) is released, usually ranging in length from (but is not limited to) 23 to 29 bases (Steps 9-11A), or the singly-nicked product containing the target product and the reverse complement of the nick site and stability region of the template (usually 36 to 48 bases in length) is released (Steps 9-11B). Another depiction of mechanisms of the reaction is presented in Figure 33.

[0090] The ratio of products 1 to 2 can be adjusted by varying the concentrations of the templates. The forward:reverse template ratio may vary from, for example, molar ratios of 100:1, 75:1; 50:1, 40:1, 30:1, 20:1, 10:1, 5:1, 2.5:1, 1:1, 1:2.5, 1:5, 1:10, 1:20, 1:30, 1:40, 1:50, 1:75, or 1:100. The ratio of products (A to B) is dependent on the ratio of nicking enzyme to polymerase, *i.e.* a higher concentration of polymerase results in more of the longer length product (B) since there is comparatively less nicking enzyme to nick both strands simultaneously before the polymerase extends. Since displaced/released product of the reverse template feeds into the forward template and vice versa, exponential amplification is achieved. The nicking enzyme:polymerase ratio may vary from, for example, enzyme unit ratios of 20:1, 15:1; 10:1, 5:1, 4:1, 3:1, 2:1, 1.5:1, 1:1, 1:1.5, 1:2, 1:3, 1:4, 1:5, 1:10, 1:15, 1:20. In certain embodiments, the ratio of nicking enzyme to polymerase may, for example, be 1:3, 1:2, 1:1.5, or 1:0.8. Those of ordinary skill in the art recognize that these ratios may represent rounded values. This nicking and polymerase extension process continues until one of the resources (usually dNTPs or enzyme) is exhausted.

[0091] As demonstrated in the Examples, the time that the reaction is run may vary from, for example, within about 1 minute, or within about 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19 or 20 minutes. Longer reaction times may produce acceptable results where speed is not an issue. In some embodiments, the reaction is between 1-20 minutes, 1-15 minutes or 1-10, 1-8, 1-5, 1-2.5, 2.5-5, 2.5-8, 2.5-10, or 2.5-20 minutes in certain embodiments. The

amplification processes described herein are efficient, and in some embodiments, as shown, for example, in the Examples, there is about $1x10^6$-fold or more amplification, about $1x10^7$-fold or more amplification, about $1x10^8$-fold or more amplification, about $1x10^9$-fold or more amplification, or about $1x10^{10}$-fold or more amplification in the time frame of the reaction, for example, in 5 or ten minutes. The reaction is highly sensitive, and is able to detect, for example, as low as about 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 20, 30, 40, 50, 60, 70, 80, 90, or 100 copies, or more, in a sample, as many as 200, 500, 1,000, 5,000, or 10,000, or more copies in a sample, or, for example, may detect a target that is present at a concentration of, for example, about 3.32E-13 micromolar to about 3.32E-8 micromolar, about 1.66E-12 micromolar to about 3.32E-8 micromolar, about 3.32E-13 micromolar to about 3.32E-7 micromolar, or about 3.32E-13 micromolar to about 3.32E-6 micromolar.

[0092] In certain exemplary embodiments, methods are provided for amplifying a double stranded nucleic acid target sequence comprising contacting a target DNA molecule comprising a double-stranded target sequence, having a sense strand and an antisense strand, with a forward template and a reverse template, wherein the forward template comprises a nucleic acid sequence comprising a recognition region at the 3' end that is complementary or substantially complementary to the 3' end of the target sequence antisense strand; a nicking enzyme binding site and a nicking site upstream of the recognition region and a stabilizing region upstream of the nicking enzyme binding site and the nicking site; the reverse template comprises a nucleotide sequence comprising a recognition region at the 3' end that is complementary or substantially complementary to the 3' end of the target sequence sense strand, a nicking enzyme binding site and a nicking site upstream of the recognition region, and a stabilizing region upstream of the nicking enzyme binding site and the nicking site; providing a first nicking enzyme that is capable of nicking at the nicking site of the forward template, and does not nick within the target sequence; providing a second nicking enzyme that is capable of nicking at the nicking site of the reverse template and does not nick within the target sequence; and providing a DNA polymerase; under conditions wherein amplification is performed by multiple cycles of the polymerase extending the forward and reverse templates along the target sequence producing a double-stranded nicking site, and the nicking enzymes nicking at the nicking sites, producing an amplification product, wherein about 10^6 (1E +06) copies of a target sequence are produced in 10 minutes, under isothermal conditions. In other embodiments, about 10^7 (1E +07) copies are produced in 10 minutes. For multiplexed assays, the time to produce the same amount of copies may be increased to about, for example, 12, 14, 15, 18, or 20 minutes. The size of the target sequence in these assays, for purposes of calculating the efficiency, may be, for example, from about 20 to about 40 nucleotides, from 20 to 30 nucleotides, or, for example, from about 20 to about 33 nucleotides. The time of the reaction is calculated from the time that all of the reaction products are present in the same vessel, container, or the like, so that the amplification reaction may start, to the time that heat is applied or chemical agents are added to stop the reaction.

[0093] In another exemplary embodiment, methods are provided for amplifying a single-stranded nucleic acid target sequence, comprising contacting a target nucleic acid comprising a single-stranded target sequence with a reverse template, wherein the reverse template comprises a nucleotide sequence comprising a recognition region at the 3' end that is complementary or substantially complementary to the 3' end of the target sequence, a nicking enzyme binding site and a nicking site upstream of the recognition region, and a stabilizing region upstream of the nicking enzyme binding site and the nicking site; providing a first nicking enzyme that is capable of nicking at the nicking site of the reverse template, and does not nick within the target sequence; providing a DNA polymerase under conditions wherein the polymerase extends the reverse template along the target sequence; contacting the extended reverse template with a forward template, wherein the forward template comprises a recognition region at the 3'end that is identical to the 5'end of the target sequence a nicking enzyme binding site and a nicking site upstream of the recognition region, and a stabilizing region upstream of the nicking enzyme binding site and the nicking site; providing a second nicking enzyme that is capable of nicking at the nicking site of the forward template and does not nick within the target sequence; under conditions wherein amplification is performed by multiple cycles of the polymerase extending the forward and reverse templates along the target sequence producing a double-stranded nicking site, and the nicking enzymes nicking at the nicking sites, producing an amplification product, wherein about 10^6 (1E +06) copies of a target sequence are produced in 10 minutes, under isothermal conditions. In other embodiments, about 10^7 (1E +07) copies are produced in 10 minutes. For multiplexed assays, the time to produce the same amount of copies may be increased to about, for example, 12, 14, 15, 18, or 20 minutes. The size of the target sequence in these assays, for purposes of calculating the efficiency, may be, for example, from about 20 to about 40 nucleotides, or, for example, from about 20 to about 33 nucleotides. The time of the reaction is calculated from the time that all of the reaction products are present in the same vessel, container, or the like, so that the amplification reaction may start.

[0094] The present methods do not require the use of temperature cycling, as often is required in methods of amplification to dissociate the target sequence from the amplified nucleic acid. The temperature of the reaction may vary based on the length of the sequence, and the GC concentration, but, as understood by those of ordinary skill in the art, the temperature should be high enough to minimize non-specific binding. The temperature should also be suitable for the enzymes of the reaction, the nicking enzyme and the polymerase. For example, the reaction may be run at about 52°C, 53°C, 54°C, 55°C, 56°C, 57°C, 58°C, 59°C, or 60°C. In some embodiments, the reaction is run at about 37°C-

85°C, 37°C -60°C, 54°C-60°C, 55°C-60°C, 58°C-60°C and, in exemplary embodiments, from 56°C-58°C. In certain embodiments, there is no denaturation step in the process. The entire amplification process, including interacting templates with target nucleic acid, is conducted within substantially isothermal conditions, and without a denaturing step (e.g., no significant temperature increase (e.g., no increase in temperature to 90-110 °C)), in some embodiments of the present methods.

[0095] Thus, in certain exemplary embodiments, methods are provided for amplifying a double stranded nucleic acid target sequence comprising contacting a target DNA molecule comprising a double-stranded target sequence, having a sense strand and an antisense strand, with a forward template and a reverse template, wherein the forward template comprises a nucleic acid sequence comprising a recognition region at the 3' end that is complementary or substantially complementary to the 3' end of the target sequence antisense strand; a nicking enzyme binding site and a nicking site upstream of the recognition region and a stabilizing region upstream of the nicking enzyme binding site and the nicking site; the reverse template comprises a nucleotide sequence comprising a recognition region at the 3' end that is complementary or substantially complementary to the 3' end of the target sequence sense strand, a nicking enzyme binding site and a nicking site upstream of the recognition region, and a stabilizing region upstream of the nicking enzyme binding site and the nicking site; providing a first nicking enzyme that is capable of nicking at the nicking site of the forward template, and does not nick within the target sequence; providing a second nicking enzyme that is capable of nicking at the nicking site of the reverse template and does not nick within the target sequence; and providing a DNA polymerase; under conditions wherein amplification is performed by multiple cycles of the polymerase extending the forward and reverse templates along the target sequence producing a double-stranded nicking site, and the nicking enzymes nicking at the nicking sites, producing an amplification product, wherein the foregoing steps are conducted under isothermal conditions.

[0096] In another exemplary embodiment, methods are provided for amplifying a single-stranded nucleic acid target sequence, comprising contacting a target nucleic acid comprising a single-stranded target sequence with a reverse template, wherein the reverse template comprises a nucleotide sequence comprising a recognition region at the 3' end that is complementary or substantially complementary to the 3' end of the target sequence, a nicking enzyme binding site and a nicking site upstream of the recognition region, and a stabilizing region upstream of the nicking enzyme binding site and the nicking site; providing a first nicking enzyme that is capable of nicking at the nicking site of the reverse template, and does not nick within the target sequence; providing a DNA polymerase under conditions wherein the polymerase extends the reverse template along the target sequence; contacting the extended reverse template with a forward template, wherein the forward template comprises a recognition region at the 3'end that is identical to the 5'end of the target sequence a nicking enzyme binding site and a nicking site upstream of the recognition region, and a stabilizing region upstream of the nicking enzyme binding site and the nicking site; providing a second nicking enzyme that is capable of nicking at the nicking site of the forward template and does not nick within the target sequence; under conditions wherein amplification is performed by multiple cycles of the polymerase extending the forward and reverse templates along the target sequence producing a double-stranded nicking site, and the nicking enzymes nicking at the nicking sites, producing an amplification product, wherein the foregoing steps are conducted under isothermal conditions.

[0097] The polymerase may be mixed with the target nucleic acid molecule before, after, or at the same time as, the nicking enzyme. In exemplary embodiments, a reaction buffer is optimized to be suitable for both the nicking enzyme and the polymerase.

[0098] Reactions may be allowed to completion, that is, when one of the resources is exhausted. Or, the reaction may be stopped using methods known to those of ordinary skill in the art, such as, for example, heat inactivation, or the addition of EDTA, high salts, or detergents. In exemplary embodiments, where mass spectrometry is to be used following amplification, EDTA may be used to stop the reaction.

Reaction Components

[0099] In a 1.5 mL Eppendorf tube combine the following reagents in order from top to bottom:

Reagent Added:	microliters Per Reaction
H_2O	31.4
10X Thermopol Buffer (NEB)	5
10X NEB Buffer 3	2.5
100 mM $MgSO_4$	4.5
10 mM dNTPs	1.5

(continued)

Reagent Added:	microliters Per Reaction
8 U/microliters Bst Pol	0.6
10 U/microliters N.BstNBI	1.5
20 micromolar Forward Template	0.25
20 micromolar Reverse Template	0.25
Total reaction mixture	47.5
Target sample	2.5
Total Reaction Volume	50 microliters

[0100] The concentrations of components for the reaction conditions in this example are as follows:

Concentration	**Component**
45.7mM	Tris-HCl
13.9 mM	KCl
10 mM	$(NH_4)_2SO_4$
50 mM	NaCl
0.5 mM	DTT
15 mM	$MgCl_2$
0.10%	Triton X-100
0.008 mM	EDTA
6 μg/mL	BSA
3.90%	Glycerol (can be lower if using a more concentrated enzyme stock)
0.3 U/microliter	Nt.BstNBI
0.1-0.4 U/microliter	Bst polymerase (large fragment)
0.1 micromolar	Forward template
0.1 micromolar	Reverse template

[0101] Variations in buffer conditions, $MgSO_4$ concentration, polymerase concentration, and template concentrations all can be optimized based on the assay sequence and desired detection method. The amount of glycerol may, for example, be lowered if a more concentrated enzyme stock is used. In certain embodiments, the concentration of Mg^{2+} ions added as a reactant is about 9mM to about 25 mM, about 9mM to 21 mM, about 9 to 21 mM, about 9 to 20 mM, about 9 to 15 mM, and, in exemplary embodiments, about 10mM to about 18 mM, about 10mM to about 25 mM, about 10mM to 21 mM, about 12 to 21 mM, about 10 to 20 mM, about 10 to 15 mM, about 10.3 mM to about 20 mM, about 10.3 mM to about 14.9 mM, or about 15 mM, for example. Also, those of ordinary skill in the art recognize that the reaction may be run without EDTA or BSA; these components may be present in the reaction as part of the storage buffers for the enzymes. The volumes can be scaled for larger or smaller total reaction volumes. The volume is usually between 5 μL and 100 μL.

[0102] The template concentrations are typically in excess of the concentration of target. The concentrations of the forward and reverse templates can be at the same or at different concentrations to bias the amplification of one product over the other. The concentration of each is usually between 10 nM and 1μM.

[0103] Additives such as BSA, non-ionic detergents such as Triton X-100 or Tween-20, DMSO, DTT, and RNase inhibitor may be included for optimization purposes without adversely affecting the amplification reaction.

Preparing/Adding Target

[0104] Targets may be diluted in 1 x Thermopol Buffer II, 1 x TE (pH 7.5) or H_20. Hot start conditions allow for faster, more specific amplification. In this case, the reaction mix (minus either enzymes or templates and target) is heated to the reaction temperature for 2 minutes, after which the reaction mix is added to the other component (enzymes or templates/target). The target can be added in any volume up to the total amount of water required in the reaction. In this

case, the target would be diluted in water. In the example above for a 50 microliter total reaction volume, 2.5 microliters of the prepared target should be added per reaction to bring the total reaction volume to 50 microliters. Reaction volumes of the present methods can be increased or decreased, depending on the needs of the user. Reaction volumes of, for example, 5, 10, 15, 20, 25, 30, 35, 40, 45, 50 microliters or more, or larger reaction volumes of, for example, 75, 100, 150, 200, 300, 400, 500 microliters, for example, may be used in the present methods.

Running the Reaction

[0105] The reaction is run at a constant temperature, usually between 54°C and 60°C for the enzyme combination of Bst polymerase (large fragment) and Nt.Bst.NB1 nicking enzyme. Other enzyme combinations may be used and the optimal reaction temperature will be based on the optimal temperature for both the nicking enzyme and polymerase to work in concert as well as the melting temperature of the reaction products. The reaction is held at temperature for 2.5 to 10 minutes, for example, until the desired amount of amplification is achieved. The reaction may be stopped by either a heat inactivation step to inactivate the enzymes (when using enzymes that can be heat-killed). Alternatively, the reaction may be stopped by adding EDTA to the reaction.

Readout

[0106] The amplified target sequence may be detected by any method known to one of ordinary skill in the art. By way of non-limiting example, several of these known methods are presented herein. In one method, amplified products may be detected by gel electrophoresis, thus detecting reaction products having a specific length. The nucleotides may, for example, be labeled, such as, for example, with biotin. Biotin-labeled amplified sequences may be captured using avidin bound to a signal generating enzyme, for example, peroxidase.

[0107] Nucleic acid detection methods may employ the use of dyes that specifically stain double-stranded DNA. Intercalating dyes that exhibit enhanced fluorescence upon binding to DNA or RNA are a basic tool in molecular and cell biology. Dyes may be, for example, DNA or RNA intercalating fluorophores and may include but are not limited to the following examples: Acridine orange, ethidium bromide, Hoechst dyes, PicoGreen, propidium iodide, SYBR I (an asymmetrical cyanine dye), SYBR II, TOTO (a thiaxole orange dimer) and YOYO (an oxazole yellow dimer), and the like. Dyes provide an opportunity for increasing the sensitivity of nucleic acid detection when used in conjunction with various detection methods and may have varying optimal usage parameters. For example ethidium bromide is commonly used to stain DNA in agarose gels after gel electrophoresis and during PCR (Hiquchi et al., Nature Biotechnology 10; 413-417, April 1992), propidium iodide and Hoechst 33258 are used in flow cytometry to determine DNA ploidy of cells, SYBR Green 1 has been used in the analysis of double-stranded DNA by capillary electrophoresis with laser induced fluorescence detection and Pico Green has been used to enhance the detection of double-stranded DNA after matched ion pair polynucleotide chromatography (Singer et al., Analytical Biochemistry 249, 229-238 1997).

[0108] Nucleic acid detection methods may also employ the use of labeled nucleotides incorporated directly into the target sequence or into probes containing complementary or substantially complementary sequences to the target of interest. Such labels may be radioactive and/or fluorescent in nature and can be resolved in any of the manners discussed herein. Labeled nucleotides, which can be detected but otherwise function as native nucleotides, are to be distinguished from modified nucleotides, which do not function as native nucleotides.

[0109] Methods of detecting and/or continuously monitoring the amplification of nucleic acid products are also well known to those skilled in the art and several examples are described below.

[0110] The production or presence of target nucleic acids and nucleic acid sequences may be detected and monitored by Molecular Beacons. Molecular Beacons are hair-pin shaped oligonucleotides containing a fluorophore on one end and a quenching dye on the opposite end. The loop of the hair-pin contains a probe sequence that is complementary or substantially complementary to a target sequence and the stem is formed by annealing of complementary or substantially complementary arm sequences located on either side of the probe sequence. A fluorophore and a quenching molecule are covalently linked at opposite ends of each arm. Under conditions that prevent the oligonucleotides from hybridizing to its complementary or substantially complementary target or when the molecular beacon is free in solution the fluorescent and quenching molecules are proximal to one another preventing fluorescence resonance energy transfer (FRET). When the molecular beacon encounters a target molecule, hybridization occurs; the loop structure is converted to a stable more rigid conformation causing separation of the fluorophore and quencher molecules leading to fluorescence (Tyagi et al. Nature Biotechnology 14: March 1996, 303-308). Due to the specificity of the probe, the generation of fluorescence is exclusively due to the synthesis of the intended amplified product.

[0111] Molecular beacons are extraordinarily specific and can discern a single nucleotide polymorphism. Molecular beacons can also be synthesized with different colored fluorophores and different target sequences, enabling several products in the same reaction to be quantified simultaneously. For quantitative amplification processes, molecular beacons can specifically bind to the amplified target following each cycle of amplification, and because non-hybridized

molecular beacons are dark, it is not necessary to isolate the probe-target hybrids to quantitatively determine the amount of amplified product. The resulting signal is proportional to the amount of amplified product. This can be done in real time. As with other real time formats, the specific reaction conditions must be optimized for each primer/probe set to ensure accuracy and precision.

[0112] The production or presence of target nucleic acids and nucleic acid sequences may also be detected and monitored by Fluorescence resonance energy transfer (FRET). FRET is an energy transfer mechanism between two chromophores: a donor and an acceptor molecule. Briefly, a donor fluorophore molecule is excited at a specific excitation wavelength. The subsequent emission from the donor molecule as it returns to its ground state may transfer excitation energy to the acceptor molecule through a long range dipole-dipole interaction. The intensity of the emission of the acceptor molecule can be monitored and is a function of the distance between the donor and the acceptor, the overlap of the donor emission spectrum and the acceptor absorption spectrum and the orientation of the donor emission dipole moment and the acceptor absorption dipole moment. FRET is a useful tool to quantify molecular dynamics, for example, in DNA-DNA interactions as seen with Molecular Beacons. For monitoring the production of a specific product a probe can be labeled with a donor molecule on one end and an acceptor molecule on the other. Probe-target hybridization brings a change in the distance or orientation of the donor and acceptor and FRET change is observed. (Joseph R. Lakowicz, "Principles of Fluorescence Spectroscopy", Plenum Publishing Corporation, 2nd edition (July 1, 1999)).

[0113] The production or presence of target nucleic acids and nucleic acid sequences may also be detected and monitored by Mass Spectrometry. Mass Spectrometry is an analytical technique that may be used to determine the structure and quantity of the target nucleic acid species and can be used to provide rapid analysis of complex mixtures. Following the method, samples are ionized, the resulting ions separated in electric and/or magnetic fields according to their mass-to-charge ratio, and a detector measures the mass-to-charge ratio of ions. (Crain, P. F. and McCloskey, J. A., Current Opinion in Biotechnology 9: 25-34 (1998)). Mass spectrometry methods include, for example, MALDI, MALDI/TOF, or Electrospray. These methods may be combined with gas chromatography (GC/MS) and liquid chromatography (LC/MS). MS has been applied to the sequence determination of DNA and RNA oligonucleotides (Limbach P., MassSpectrom. Rev. 15: 297-336 (1996); Murray K., J. Mass Spectrom. 31: 1203-1215 (1996)). MS and more particularly, matrix-assisted laser desorption/ionization MS (MALDI MS) has the potential of very high throughput due to high-speed signal acquisition and automated analysis off solid surfaces. It has been pointed out that MS, in addition to saving time, measures an intrinsic property of the molecules, and therefore yields a significantly more informative signal (Koster H. et al., Nature Biotechnol., 14: 1123-1128 (1996)).

[0114] The production or presence of target nucleic acids and nucleic acid sequences may also be detected and monitored by various methods of gel electrophoresis. Gel electrophoresis involves the separation of nucleic acids through a matrix, generally a cross-linked polymer, using an electromotive force that pulls the molecules through the matrix. Molecules move through the matrix at different rates causing a separation between products that can be visualized and interpreted via any one of a number of methods including but not limited to; autoradiography, phosphorimaging, and staining with nucleic acid chelating dyes.

[0115] The production or presence of target nucleic acids and nucleic acid sequences may also be detected and monitored by capillary gel electrophoresis. Capillary-gel Electrophoresis (CGE) is a combination of traditional gel electrophoresis and liquid chromatography that employs a medium such as polyacrylamide in a narrow bore capillary to generate fast, high-efficient separations of nucleic acid molecules with up to single base resolution. CGE is commonly combined with laser induced fluorescence (LIF) detection where as few as six molecules of stained DNA can be detected. CGE/LIF detection generally involves the use of fluorescent DNA intercalating dyes including ethidium bromide, YOYO and SYBR Green 1 but can also involve the use of fluorescent DNA derivatives where the fluorescent dye is covalently bound to the DNA. Simultaneous identification of several different target sequences can be made using this method.

[0116] The production or presence of target nucleic acids and nucleic acid sequences may also be detected and monitored by various surface capture methods. This is accomplished by the immobilization of specific oligonucleotides to a surface producing a biosensor that is both highly sensitive and selective. Surfaces used in this method may include but are not limited to gold and carbon and may use a number of covalent or noncovalent coupling methods to attach the probe to the surface. The subsequent detection of a target DNA can be monitored by a variety of methods.

[0117] Electrochemical methods generally involve measuring the cathodic peak of intercalators, such as methylene blue, on the DNA probe electrode and visualized with square wave voltammograms. Binding of the target sequence can be observed by a decrease in the magnitude of the voltammetric reduction signals of methylene blue as it interacts with dsDNA and ssDNA differently reflecting the extent of the hybrid formation.

[0118] Surface Plasmon Resonance (SPR) can also be used to monitor the kinetics of probe attachment as well as the process of target capture. SPR does not require the use of fluorescence probes or other labels. SPR relies on the principle of light being reflected and refracted on an interface of two transparent media of different refractive indexes. Using monochromatic and p-polarized light and two transparent media with an interface comprising a thin layer of gold, total reflection of light is observed beyond a critical angle, however the electromagnetic field component of the light penetrates into the medium of lower refractive index creating an evanescent wave and a sharp shadow (surface plasmon

resonance). This is due to the resonance energy transfer between the wave and the surface plasmons. The resonance conditions are influenced by the material absorbed on the thin metal film and nucleic acid molecules, proteins and sugars concentrations are able to be measured based on the relation between resonance units and mass concentration.

[0119] The production or presence of target nucleic acids and nucleic acid sequences may also be detected and monitored by lateral flow devices. Lateral Flow devices are well known. These devices generally include a solid phase fluid permeable flow path through which fluid flows through by capillary force. Examples include, but are not limited to, dipstick assays and thin layer chromatographic plates with various appropriate coatings. Immobilized on the flow path are various binding reagents for the sample, binding partners or conjugates involving binding partners for the sample and signal producing systems. Detection of samples can be achieved in several manners; enzymatic detection, nanoparticle detection, colorimetric detection, and fluorescence detection, for example. Enzymatic detection may involve enzyme-labeled probes that are hybridized to complementary or substantially complementary nucleic aid targets on the surface of the lateral flow device. The resulting complex can be treated with appropriate markers to develop a readable signal. Nanoparticle detection involves bead technology that may use colloidal gold, latex and paramagnetic nanoparticles. In one example, beads may be conjugated to an anti-biotin antibody. Target sequences may be directly biotinylated, or target sequences may be hybridized to a sequence specific biotinylated probes. Gold and latex give rise to colorimetric signals visible to the naked eye and paramagnetic particles give rise to a non-visual signal when excited in a magnetic field and can be interpreted by a specialized reader.

[0120] Fluorescence-based lateral flow detection methods are also known, for example, dual fluorescein and biotin-labeled oligo probe methods, UPT-NALF utilizing up-converting phosphor reporters composed of lanthanide elements embedded in a crystal (Corstjens et al., Clinical Chemistry, 47:10, 1885-1893, 2001), as well as the use of quantum dots.

[0121] Nucleic acids can also be captured on lateral flow devices. Means of capture may include antibody dependent and antibody independent methods. Antibody-dependent capture generally comprises an antibody capture line and a labeled probe that is complementary or substantially complementary sequence to the target. Antibody-independent capture generally uses non-covalent interactions between two binding partners, for example, the high affinity and irreversible linkage between a biotinylated probe and a streptavidin line. Capture probes may be immobilized directly on lateral flow membranes. Both antibody dependent and antibody independent methods may be used in multiplexing.

[0122] The production or presence of target nucleic acids and nucleic acid sequences may also be detected and monitored by multiplex DNA sequencing. Multiplex DNA sequencing is a means of identifying target DNA sequences from a pool of DNA. The technique allows for the simultaneous processing of many sequencing templates. Pooled multiple templates can be resolved into individual sequences at the completion of processing. Briefly, DNA molecules are pooled, amplified and chemically fragmented. Products are fractionated by size on sequencing gels and transferred to nylon membranes. The membranes are probed and autoradiographed using methods similar to those used in standard DNA sequencing techniques (Church et al., Science 1998 Apr 8;240(4849):185-188). Autoradiographs can be evaluated and the presence of target nucleic acid sequence can be quantified.

Kits

[0123] Kits used for the present methods may comprise, for example, one or more polymerases, forward and reverse templates, and one or more nicking enzymes, as described herein. Where one target is to be amplified, one or two nicking enzymes may be included in the kit. Where multiple target sequences are to be amplified, and the templates designed for those target sequences comprise the nicking enzyme binding sites for the same nicking enzyme, then one or two nicking enzymes may be included. Or, where the templates are recognized by different nicking enzymes, more nicking enzymes may be included in the kit, such as, for example, 3 or more.

[0124] The kits used for the present methods may also comprise one or more of the components in any number of separate containers, packets, tubes, vials, microtiter plates and the like, or the components may be combined in various combinations in such containers.

[0125] The components of the kit may, for example, be present in one or more containers, for example, all of the components may be in one container, or, for example, the enzymes may be in a separate container from the templates. The components may, for example, be lyophilized, freeze dried, or in a stable buffer. In one example, the polymerase and nicking enzymes are in lyophilized form in a single container, and the templates are either lyophilized, freeze dried, or in buffer, in a different container. Or, in another example, the polymerase, nicking enzymes, and the templates are, in lyophilized form, in a single container. Or, the polymerase and the nicking enzyme may be separated into different containers.

[0126] Kits may further comprise, for example, dNTPs used in the reaction, or modified nucleotides, cuvettes or other containers used for the reaction, or a vial of water or buffer for re-hydrating lyophilized components. The buffer used may, for example, be appropriate for both polymerase and nicking enzyme activity.

[0127] The kits used for the present methods may also comprise instructions for performing one or more methods described herein and/or a description of one or more compositions or reagents described herein. Instructions and/or

descriptions may be in printed form and may be included in a kit insert. A kit also may include a written description of an Internet location that provides such instructions or descriptions.

[0128] Kits may further comprise reagents used for detection methods, such as, for example, reagents used for FRET, lateral flow devices, dipsticks, fluorescent dye, colloidal gold particles, latex particles, a molecular beacon, or polystyrene beads.

[0129] An advantage of the present methods and the present kits is that they can be used in any device that provides a constant temperature, including thermocyclers, incubation ovens, water baths, and heat blocks.

[0130] Thus, provided in the present methods is method for nucleotide sequence amplification, which comprises: combining a target nucleic acid having a target nucleotide sequence with (i) a polymerase, (ii) a first template nucleic acid that hybridizes to the a first strand of the target nucleotide sequence, and (iii) a second template nucleic acid that hybridizes to the complement of the first strand of the target nucleotide sequence, in an amplification reaction, under conditions in which the polymerase extends the template nucleic acids, thereby generating extended template nucleic acid amplicons; wherein:the target nucleotide sequence is between 20 and 40 nucleotides in length; the target nucleotide sequence is amplified 1E+6-fold or more in about ten minutes; and the foregoing steps are conducted under substantially isothermal conditions.

[0131] Also provided is a method for nucleotide sequence amplification, which comprises: combining a target nucleic acid having a target nucleotide sequence with (i) a polymerase, (ii) a first template nucleic acid that hybridizes to the a first strand of the target nucleotide sequence, and (iii) a second template nucleic acid that hybridizes to the complement of the first strand of the target nucleotide sequence, in an amplification reaction, under conditions in which the polymerase extends the template nucleic acids, thereby generating extended template nucleic acid amplicons; wherein:the target nucleotide sequence is between 20 and 40 nucleotides in length; the first template comprises a nucleic acid sequence comprising a first template recognition region at the 3' end that is complementary or substantially complementary to the 3' end of the first strand of the target nucleotide sequence; the second template comprises a nucleotide sequence comprising a second template recognition region at the 3' end that is complementary or substantially complementary to the 3' end of the complement of the first strand of the target nucleotide sequence; the target nucleotide sequence comprises from 1 to 5 nucleotides more than the sum of the nucleotides of the first template recognition region and the second template recognition region; the target nucleotide sequence is amplified 1E+6-fold or more in about ten minutes; and the foregoing steps are conducted under substantially isothermal conditions.

[0132] Also provided is a method for nucleotide sequence amplification, which comprises: combining a target nucleic acid having a target nucleotide sequence with (i) a polymerase, (ii) a first template nucleic acid that hybridizes to the a first strand of the target nucleotide sequence, and (iii) a second template nucleic acid that hybridizes to the complement of the first strand of the target nucleotide sequence, in an amplification reaction, under conditions in which the polymerase extends the template nucleic acids, thereby generating extended template nucleic acid amplicons; wherein:the first template comprises a nucleic acid sequence comprising a first template recognition region at the 3' end that is complementary or substantially complementary to the 3' end of the first strand of the target nucleotide sequence, wherein the recognition region is 8-15 nucleotides long; the second template comprises a nucleotide sequence comprising a second template recognition region at the 3' end that is complementary or substantially complementary to the 3' end of the complement of the first strand of the target nucleotide sequence, wherein the recognition region is 8-15 nucleotides long; the target nucleotide sequence is amplified 1E+6-fold or more in about ten minutes; and the foregoing steps are conducted under substantially isothermal conditions.

[0133] In certain aspects of the present methods, the first template comprises a nucleic acid sequence comprising a first template recognition region at the 3' end that is complementary or substantially complementary to the 3' end of the first strand of the target nucleotide sequence; and the second template comprises a nucleotide sequence comprising a second template recognition region at the 3' end that is complementary or substantially complementary to the 3' end of the complement of the first strand of the target nucleotide sequence. In certain aspects, the target nucleotide sequence comprises from 1 to 5 nucleotides more than the sum of the nucleotides of the first template recognition region and the second template recognition region. In other aspects, the first template and second templates comprise nicking enzyme binding sites and nicking sites upstream of the recognition regions, and the amplification reaction further comprises one or more nicking enzymes that are capable of nicking at the nicking site of said forward and said reverse templates, wherein either one nicking enzyme is capable of nicking both of said templates, or each template is capable of being nicked by at least one of the nicking enzymes, and wherein said one or more nicking enzymes do not nick within said target sequence.

[0134] In some embodiments, the target nucleotide sequence comprises 1 nucleotide more than the sum of the nucleotides of the first template recognition region and the second template recognition region. In other embodiments, the target nucleotide sequence comprises 2 nucleotides more than the sum of the nucleotides of the first template recognition region and the second template recognition region. In yet other embodiments, the target nucleotide sequence comprises 3 nucleotides more than the sum of the nucleotides of the first template recognition region and the second template recognition region.

[0135] In certain aspects of the present methods, the target nucleic acid is double stranded or single stranded. In certain aspects, the target nucleic acid is double-stranded DNA. In other aspects, the target nucleic acid is single-stranded DNA. In yet other aspects, the target nucleic acid is RNA. The target nucleic acid may be, for example, selected from the group consisting of genomic DNA, plasmid DNA, viral DNA, mitochondrial DNA, and synthetic double-stranded DNA. The target nucleic acid may be, for example, selected from the group consisting of viral DNA, cDNA, and synthetic single-stranded DNA. The target nucleic acid may be, for example, selected from the group consisting of messenger RNA, viral RNA, ribosomal RNA, transfer RNA, micro RNA, micro RNA precursor, and synthetic RNA.

[0136] In the present methods, the DNA polymerase may be, for example, a thermophilic polymerase. The polymerase may, for example, be selected from the group consisting of Bst (large fragment), 9°N, $Vent_R$® (exo-) DNA Polymerase, Therminator, and Therminator II. In certain aspects, the polymerase is Bst (large fragment).

[0137] In certain embodiments, the first and second templates comprise nicking enzyme binding sites recognized by the same nicking enzyme and said first and said second nicking enzyme are the same. The nicking enzymes may be, for example, selected from the group consisting of Nt.BspQI, Nb.BbvCi, Nb.BsmI, Nb.BsrDI, Nb.BtsI, Nt.AlwI, Nt.BbvCI, Nt.BstNBI, Nt.CviPII, Nb.Bpu10I, and Nt.Bpu10I.

[0138] In some aspects of the present method, the portion of the nucleic acid sequence of the first strand that is complementary or substantially complementary to the first strand of the target nucleotide sequence is 8-15 nucleotides in length and wherein the portion of the second strand that is complementary or substantially complementary to the target nucleotide sequence is 8-15 nucleotides in length. In some aspects, the first template is provided at the same concentration as the second template. In other aspects, one of the first or second templates is provided at a ratio to the other template at the range of ratios of 1:100 to 100:1. The reactions of the present methods may further comprise a second polymerase. In some aspects, at least one of the first or second polymerases comprises reverse transcriptase activity.

[0139] In certain embodiments of the present method, the amplification is conducted between 54°C and 60°C. In other embodiments, the amplification is conducted between 56°C and 58°C. In certain embodiments, wherein the amplification reaction is held at a constant temperature for 1 to 10 minutes. In other embodiments, the amplification reaction is held at a constant temperature for 1 to 20 minutes.

[0140] The present method may further comprise detecting the amplification product. Thus, in certain aspects, the amplification product is detected by detection method selected from the group consisting of gel electrophoresis, mass spectrometry, SYBR I fluorescence, SYBR II fluorescence, SYBR Gold, Pico Green, TOTO-3, intercalating dye detection, fluorescence resonance energy transfer (FRET), molecular beacon detection, surface capture, capillary electrophoresis, incorporation of labeled nucleotides to allow detection by capture, fluorescence polarization, and lateral flow capture.

[0141] In some aspects, at least two target sequences are capable of being amplified. In certain aspects, the amplification products are detected on a solid surface. In some aspects, at least one capture probe is immobilized on a solid surface. In some embodiments, at least one of said templates comprises a spacer, blocking group, or a modified nucleotide.

[0142] In certain embodiments of the present methods, the target nucleotide sequence is amplified 1E+6-fold or more in about five minutes. In other embodiments, the target nucleotide sequence is amplified 1E+6-fold or more in about 2.5 minutes. In other embodiments, the target nucleotide sequence is amplified 1E+7-fold or more in about five minutes. In other embodiments, the target nucleotide sequence is amplified 1E+8-fold or more in about five minutes. In yet other embodiments, wherein the target nucleotide sequence is amplified 1E+9-fold or more in about five minutes.

[0143] The present methods also include a method for amplifying a double-stranded nucleic acid target sequence, comprising contacting a target DNA molecule comprising a double-stranded target sequence, having a sense strand and an antisense strand, with a forward template and a reverse template, wherein said forward template comprises a nucleic acid sequence comprising a recognition region at the 3' end that is complementary to the 3' end of the target sequence antisense strand; a nicking enzyme binding site and a nicking site upstream of said recognition region and a stabilizing region upstream of said nicking site, wherein the portion of the nucleic acid sequence that is complementary to the 3' end of the target antisense strand is 8-15 nucleotides in length; said reverse template comprises a nucleotide sequence comprising recognition region at the 3' end that is complementary to the 3' end of the target sequence sense strand, a nicking enzyme binding site and a nicking site upstream of said recognition region, and a stabilizing region upstream of said nicking site, wherein the portion of the nucleic acid sequence that is complementary to the 3' end of the target antisense strand is 8-15 nucleotides in length; providing a first nicking enzyme that is capable of nicking upstream, downstream, or at the nicking site of said forward template, and does not nick within said target sequence; providing a second nicking enzyme that is capable of nicking upstream, downstream, or at the nicking site of said reverse template and does not nick within said target sequence; and providing a DNA polymerase;
under essentially isothermal conditions, wherein amplification is performed by multiple cycles of said polymerase extending said forward and reverse templates along said target sequence producing a double-stranded nicking site, and said nicking enzymes nicking at said nicking sites, or amplified copies of said sites, producing an amplification product.

[0144] Also provided is a method for amplifying a single-stranded nucleic acid target sequence, comprising contacting

a target nucleic acid comprising a single-stranded target sequence with a reverse template, wherein said reverse template comprises a nucleic acid sequence comprising a recognition region at the 3' end that is complementary to the 3' end of the target sequence, a nicking enzyme binding site and a nicking site upstream of said recognition region, and a stabilizing region upstream of said nicking site, wherein the portion of the nucleic acid sequence that is complementary to the 3' end of the target sequence is 8-15 nucleotides in length; providing a first nicking enzyme that is capable of nicking at the nicking site of said reverse template, and does not nick within said target sequence; providing a DNA polymerase under conditions wherein said polymerase extends said reverse template along said target sequence; contacting said extended reverse template with a forward template, wherein said forward template comprises a recognition region at the 3'end that is complementary to the 3' end of the extended reverse template, a nicking enzyme binding site and a nicking site upstream of said recognition region, and a stabilizing region upstream of said nicking site, wherein the portion of the nucleic acid sequence that is complementary to the 3' end of the target antisense strand is 8-15 nucleotides in length; providing a second nicking enzyme that is capable of nicking at the nicking site of said forward template and does not nick within said target sequence or within the complement of said target sequence; wherein the amplification is conducted under essentially isothermal conditions wherein amplification is performed by multiple cycles of said polymerase extending said forward and reverse templates along said target sequence, producing double-stranded nicking sites, and said nicking enzymes nicking at said nicking sites, producing an amplification product. In some aspects of the present method, the DNA polymerase is a thermophilic polymerase. For example, the polymerase may be selected from the group consisting of Bst (large fragment), 9°N, $Vent_R$® (exo-) DNA Polymerase, Therminator, and Therminator II. In certain aspects, the polymerase is Bst(large fragment).

[0145] In certain aspects, the nicking enzymes nick downstream of the nicking enzyme binding site. In other aspects, the forward and reverse templates comprise nicking enzyme binding sites recognized by the same nicking enzyme and said first and said second nicking enzymes are the same. In certain aspects, the nicking enzymes are selected from the group consisting of Nt.BspQI, Nb.BbvCi, Nb.BsmI, Nb.BsrDI, Nb.BtsI, Nt.AlwI, Nt.BbvCI, Nt.BstNBI, Nt.CviPII, Nb.Bpu10I, and Nt.Bpu10I.

[0146] In some embodiments of the present methods, the target sequence comprises from 1 to 5 nucleotides more than the sum of the nucleotides of said forward template recognition region and said reverse template recognition region. In certain embodiments, the target sequence comprises 1 nucleotide more than the sum of the nucleotides of said forward template recognition region and said reverse template recognition region. In other embodiments, the target sequence comprises 2 nucleotides more than the sum of the nucleotides of said forward template recognition region and said reverse template recognition region.

[0147] In certain aspects, the target DNA molecule is selected from the group consisting of genomic DNA, plasmid, mitochondrial, and viral DNA. In other aspects, the target nucleic acid is selected from the group consisting of viral DNA, messenger RNA, microRNA, and microRNA precursors. In other aspects, the forward template is provided at the same concentration as the reverse template. In yet other aspects, one of the forward or reverse templates is provided at a ratio to the other template at the range of ratios of 1:100 to 100:1

[0148] In certain embodiments, the present method further comprises a second polymerase. For example, at least one of the polymerases may comprise reverse transcriptase activity. In certain aspects, the amplification is conducted between 54°C and 60°C. In other aspects, the amplification reaction is held at a constant temperature for 1 to 10 minutes.

[0149] The present method may further comprise detecting the amplification product. For example, the amplification product may be detected by a method selected from the group consisting of gel electrophoresis, mass spectrometry, SYBR I fluorescence, SYBR II fluorescence, SYBR Gold, Pico Green, TOTO-3, intercalating dye detection, FRET, molecular beacon detection, surface capture, capillary electrophoresis, incorporation of labeled nucleotides to allow detection by capture, fluorescence polarization, and lateral flow capture.

[0150] In certain aspects, at least two target sequences are capable of being amplified. In other aspects, the amplification products are detected on a solid surface. In some aspects, at least one capture probe is immobilized on a solid surface. In other aspects, at least one of said templates comprises a spacer, blocking group, or a modified nucleotide.

[0151] The present methods also include a method for amplifying a double-stranded nucleic acid target sequence, comprising contacting a target DNA molecule comprising a double-stranded target sequence, having a sense strand and an antisense strand, with a forward template and a reverse template, wherein said forward template comprises a nucleic acid sequence comprising a recognition region at the 3' end that is complementary to the 3' end of the target sequence antisense strand; a nicking enzyme binding site and a nicking site upstream of said recognition region and a stabilizing region upstream of said nicking site; said reverse template comprises a nucleotide sequence comprising a recognition region at the 3' end that is complementary to the 3' end of the target sequence sense strand, a nicking enzyme binding site and a nicking site upstream of said recognition region, and a stabilizing region upstream of said nicking site; and said target sequence comprises from 1 to 5 nucleotides more than the sum of the nucleotides of said forward template recognition region and said reverse template recognition region; providing a first nicking enzyme that is capable of nicking at the nicking site of said forward template, and does not nick within said target sequence; providing a second nicking enzyme that is capable of nicking at the nicking site of said reverse template and does not nick within

said target sequence; and providing a DNA polymerase; under essentially isothermal conditions, wherein amplification is performed by multiple cycles of said polymerase extending said forward and reverse templates along said target sequence producing a double-stranded nicking site, and said nicking enzymes nicking at said nicking sites, or amplified copies of said sites, producing an amplification product.

5 **[0152]** Also provided is a method for amplifying a single-stranded nucleic acid target sequence, comprising contacting a target nucleic acid comprising a single-stranded target sequence with a reverse template, wherein said reverse template comprises a nucleic acid sequence comprising a recognition region at the 3' end that is complementary to the 3' end of the target sequence, a nicking enzyme binding site and a nicking site upstream of said recognition region, and a stabilizing region upstream of said nicking site, wherein the portion of the nucleic acid sequence that is complementary to the 3'

10 end of the target sequence is 8-15 nucleotides in length; providing a first nicking enzyme that is capable of nicking at the nicking site of said reverse template, and does not nick within said target sequence; providing a DNA polymerase under conditions wherein said polymerase extends said reverse template along said target sequence; contacting said extended reverse template with a forward template, wherein said forward template comprises a recognition region at the 3'end that is complementary to the 3' end of the extended reverse template, a nicking enzyme binding site and a

15 nicking site upstream of said recognition region, and a stabilizing region upstream of said nicking site, wherein said target sequence comprises from 1 to 5 nucleotides more than the sum of the nucleotides of said forward template recognition region and said reverse template recognition region; providing a second nicking enzyme that is capable of nicking at the nicking site of said forward template and does not nick within said target sequence or within the complement of said target sequence; wherein the amplification is conducted under essentially isothermal conditions wherein ampli-

20 fication is performed by multiple cycles of said polymerase extending said forward and reverse templates along said target sequence, producing double-stranded nicking sites, and said nicking enzymes nicking at said nicking sites, producing an amplification product.

[0153] In certain embodiments, the target sequence comprises 1 nucleotide more than the sum of the nucleotides of said forward template recognition region and said reverse template recognition region. In other embodiments, the target

25 sequence comprises 2 nucleotides more than the sum of the nucleotides of said forward template recognition region and said reverse template recognition region. In other embodiments, the target sequence comprises 3 nucleotides more than the sum of the nucleotides of said forward template recognition region and said reverse template recognition region.

[0154] Also provided is a method for amplifying a double-stranded nucleic acid target sequence, comprising contacting a target DNA molecule comprising a double-stranded target sequence, having a sense strand and an antisense strand,

30 with a forward template and a reverse template, wherein said forward template comprises a nucleic acid sequence comprising a recognition region at the 3' end that is complementary to the 3' end of the target sequence antisense strand; a nicking enzyme binding site and a nicking site upstream of said recognition region and a stabilizing region upstream of said nicking site; said reverse template comprises a nucleotide sequence comprising a recognition region at the 3' end that is complementary to the 3' end of the target sequence sense strand, a nicking enzyme binding site and a nicking

35 site upstream of said recognition region, and a stabilizing region upstream of said nicking site; providing a first nicking enzyme that is capable of nicking at the nicking site of said forward template, and does not nick within said target sequence; providing a second nicking enzyme that is capable of nicking at the nicking site of said reverse template and does not nick within said target sequence; and providing a DNA polymerase; under essentially isothermal conditions, wherein amplification is performed by multiple cycles of said polymerase extending said forward and reverse templates

40 along said target sequence producing a double-stranded nicking site, and said nicking enzymes nicking at said nicking sites, or amplified copies of said sites, producing an amplification product.

[0155] Also provided is method for amplifying a double-stranded nucleic acid target sequence, comprising contacting a target DNA molecule comprising a double-stranded target sequence, having a sense strand and an antisense strand, with a forward template and a reverse template, wherein said forward template comprises a nucleic acid sequence

45 comprising a recognition region at the 3' end that is complementary to the 3' end of the target sequence antisense strand; a nicking enzyme binding site and a nicking site upstream of said recognition region and a stabilizing region upstream of said nicking site; said reverse template comprises a nucleotide sequence comprising recognition region at the 3' end that is complementary to the 3' end of the target sequence sense strand, a nicking enzyme binding site and a nicking site upstream of said recognition region, and a stabilizing region upstream of said nicking site; providing a first nicking

50 enzyme that is capable of nicking upstream, downstream, or at the nicking site of said forward template, and does not nick within said target sequence; providing a second nicking enzyme that is capable of nicking upstream, downstream, or at the nicking site of said reverse template and does not nick within said target sequence; and providing a DNA polymerase;under essentially isothermal conditions, wherein amplification is performed by multiple cycles of said polymerase extending said forward and reverse templates along said target sequence producing a double-stranded

55 nicking site, and said nicking enzymes nicking at said nicking sites, or amplified copies of said sites, producing an amplification product.

[0156] Also provided is a method for amplifying a double-stranded nucleic acid target sequence, comprising contacting a target DNA molecule comprising a double-stranded target sequence, having a sense strand and an antisense strand,

with a forward template and a reverse template, wherein said forward template comprises a nucleic acid sequence comprising a recognition region at the 3' end that is complementary to the 3' end of the target sequence antisense strand; a nicking enzyme binding site and a nicking site upstream of said recognition region and a stabilizing region upstream of said nicking site; said reverse template comprises a nucleotide sequence comprising a recognition region at the 3' end that is complementary to the 3' end of the target sequence sense strand, a nicking enzyme binding site and a nicking site upstream of said recognition region, and a stabilizing region upstream of said nicking site; providing a first nicking enzyme that is capable of nicking at the nicking site of said forward template, and does not nick within said target sequence; providing a second nicking enzyme that is capable of nicking at the nicking site of said reverse template and does not nick within said target sequence; and providing a DNA polymerase; under essentially isothermal conditions, wherein amplification is performed by multiple cycles of said polymerase extending said forward and reverse templates along said target sequence producing a double-stranded nicking site, and said nicking enzymes nicking at said nicking sites, or amplified copies of said sites, producing an amplification product, wherein at least a 1E + 7 fold amplification of a 22-35 nucleotide long target sequence is obtained when the amplification reaction is run for twelve minutes.

[0157] The present method also provides a method for amplifying a double-stranded nucleic acid target sequence, comprising contacting a target DNA molecule comprising a double-stranded target sequence, having a sense strand and an antisense strand, with a forward template and a reverse template, wherein said forward template comprises a nucleic acid sequence comprising a recognition region at the 3' end that is complementary to the 3' end of the target sequence antisense strand; a nicking enzyme binding site and a nicking site upstream of said recognition region and a stabilizing region upstream of said nicking site, wherein the portion of the nucleic acid sequence that is complementary to the 3' end of the target antisense strand is 8-15 nucleotides in length; said reverse template comprises a nucleotide sequence comprising recognition region at the 3' end that is complementary to the 3' end of the target sequence sense strand, a nicking enzyme binding site and a nicking site upstream of said recognition region, and a stabilizing region upstream of said nicking site, wherein the portion of the nucleic acid sequence that is complementary to the 3' end of the target antisense strand is 8-15 nucleotides in length; providing a first nicking enzyme that is capable of nicking upstream, downstream, or at the nicking site of said forward template, and does not nick within said target sequence; providing a second nicking enzyme that is capable of nicking upstream, downstream, or at the nicking site of said reverse template and does not nick within said target sequence; and providing a DNA polymerase; under essentially isothermal conditions, wherein amplification is performed by multiple cycles of said polymerase extending said forward and reverse templates along said target sequence producing a double-stranded nicking site, and said nicking enzymes nicking at said nicking sites, or amplified copies of said sites, producing an amplification product, wherein at least a 1E + 7 fold amplification of a 22-35 nucleotide long target sequence is obtained when the amplification reaction is run for twelve minutes.

[0158] Also provided are kits for amplifying a nucleic acid target sequence, comprising a DNA polymerase; a first template for nucleic acid amplification, comprising a recognition region at the 3' end that is complementary to the 3' end of a target sequence sense strand; a nicking enzyme binding site and a nicking site upstream of said recognition region; and a stabilizing region upstream of said nicking site, wherein the portion of the nucleic acid sequence that is complementary to the 3' end of the target sequence sense strand is 8-15 nucleotides in length; a second template for nucleic acid amplification, comprising a recognition region at the 3' end that is complementary to the 3' end of the complement of the target sequence sense strand; a nicking enzyme binding site and a nicking site upstream of said recognition region; and a stabilizing region upstream of said nicking site, wherein the portion of the nucleic acid sequence that is complementary to the 3' end of the complement of the target sequence sense strand is 8-15 nucleotides in length; and one or two thermostable nicking enzymes, wherein either one enzyme is capable of nicking at the nicking site of said first and said second templates, or a first enzyme is capable of nicking at the nicking site of said first primer and a second enzyme is capable of nicking at the enzyme site of said second primer.

[0159] In certain embodiments, the target sequence comprises from 1 to 5 nucleotides more than the sum of the nucleotides of said first template recognition region and said second template recognition region. In certain embodiments, the polymerase, nicking enzymes, and templates are in a container. In certain embodiments, the polymerase, nicking enzymes, and templates are in two containers. In other embodiments, the polymerase and nicking enzymes are in a first container, and said templates are in a second container. In some aspects, the polymerase, nicking enzymes, and templates are lyophilized. In some aspects, the kits further comprise instructions for following the method of amplification. The kits may further comprise a cuvette. Or, for example, the kits may further comprise a lateral flow device or dipstick. In some aspects, the lateral flow device or dipstick further comprises a capture probe. In some aspects, the kit further comprises a detector component selected from the group consisting of a fluorescent dye, colloidal gold particles, latex particles, a molecular beacon, and polystyrene beads. In some aspects of the kit, at least one of said templates comprises a spacer, blocking group, or a modified nucleotide.

[0160] Also provided is a kit for amplifying a nucleic acid target sequence, comprising a DNA polymerase; a first template for nucleic acid amplification, comprising a recognition region at the 3' end that is complementary to the 3' end of a target sequence sense strand; a nicking enzyme binding site and a nicking site upstream of said recognition region;

and a stabilizing region upstream of said nicking site; a second template for nucleic acid amplification, comprising a recognition region at the 3' end that is complementary to the 3' end of the complement of the target sequence sense strand; a nicking enzyme binding site and a nicking site upstream of said recognition region; and a stabilizing region upstream of said nicking site, wherein said target sequence comprises from 1 to 5 nucleotides more than the sum of the nucleotides of said first template recognition region and said second template recognition region; and one or two thermostable nicking enzymes, wherein either one enzyme is capable of nicking at the nicking site of said first and said second templates, or a first enzyme is capable of nicking at the nicking site of said first primer and a second enzyme is capable of nicking at the enzyme site of said second primer. In certain aspects of the kit, the portion of the nucleic acid sequence of the first template that is complementary to the 3' end of the target sequence sense strand is 8-15 nucleotides in length, and the portion of the nucleic acid sequence of the second template that is complementary to the 3' end of the complement of the target sequence sense strand is 8-15 nucleotides in length.

[0161] In certain embodiments, the polymerase, nicking enzymes, and templates are in two containers. In other embodiments, the polymerase and nicking enzymes are in a first container, and said templates are in a second container. In some aspects, the polymerase, nicking enzymes, and templates are lyophilized. In some aspects, the kits further comprise instructions for following the method of amplification. The kits may further comprise a cuvette. Or, for example, the kits may further comprise a lateral flow device or dipstick. In some aspects, the lateral flow device or dipstick further comprises a capture probe. In some aspects, the kit further comprises a detector component selected from the group consisting of a fluorescent dye, colloidal gold particles, latex particles, a molecular beacon, and polystyrene beads. In some aspects of the kit, at least one of said templates comprises a spacer, blocking group, or a modified nucleotide.

Examples

Example 1: Sample NEAR™ Amplification Assay

[0162] This example provides an example of a typical DNA wet assay of the present method. Those of ordinary skill in the art understand that numerous modifications may be made to the volumes and format of the reaction, the length of time that the assay is conducted, and the amounts of each reactant.

[0163] Two 96-well microtiter plates are used to set up "wet" assays, a Template/Target plate and a Master Mix plate. To begin, 5 microliters of templates are aliquoted into appropriate wells on the Template Target plate. For the "- target" wells (control wells without target), 5 microliters of dH_2O are added. A reagent master mix is created by combining buffer, salt, dNTPs, enzymes, and dH_2O together in a single tube, using appropriate volumes of each based on the number of samples being tested (see Table within this Example). 40 microliters of reagent master mix is aliquotted into both "-target" and "+ target" (control wells with target) wells of the Master Mix plate, and the plate is sealed with thermal sealant. All of the previous steps were completed in a pre-amplification room with all of the subsequent steps completed in a post-amplification room. The thermal sealant is removed from the Template/Target plate, from only the wells that target will be added to, leaving the "- wells" sealed to avoid potential contamination. 5 microliters of target is aliquoted into the appropriate "+ target" wells. The Template/Target plate is resealed with thermal sealant. Both the Template/Target plate and Master Mix plate are incubated for 2-3 minutes at assay temperature (for example, at 56°C, 57°C, or 58°C, using thermal cyclers. The thermal sealant is removed from both plates. 40 microliters of reagent master mix from the Master Mix plate wells is transferred to the appropriate wells on the Template/Target plate, and the Template/Target plate is resealed with thermal sealant. The samples are incubated for 5-10 minutes at assay temperature. The time for the reaction is calculated from the time that the incubation starts, immediately after the reagent master mix if transferred to the wells on the Template/Target plate, the plate is sealed, and placed in the thermocycler. Reactions are stopped by adding SDS to 0.1% or greater, or by incubating the samples for 2 minutes at 80°C.

[0164] To detect the amplified products, for example, 3-5 microliters of 5 micromolar molecular beacon is added to each well and mixed by pipetting up and down several times. A fluorescence read is performed at the appropriate wavelength based on the fluorophone present on the molecular beacon, at assay temperature, following a 1 minute incubation

Typical reagent breakdown for single 50 microliter DNA reactions (all volumes in microliters)

[0165]

Reagent	- Target	+ target	Final Concentration
5XIB2 Buffer	10.0	10.0	1X
100 mM $MgSO_4$	2.5	2.5	10+ 5mM

(continued)

Reagent	- Target	+ target	Final Concentration
10mM dNTPs	1.5	1.5	0.3 mM
8 U/microliter Bst Pol	2.4	2.4	19.2 units
10 U/microliter N. BstNB1	1.50	1.50	15 units
Template 1	2.5	2.5	10-1000 nM
Template 2	2.5	2.5	10-1000 nM
Target	0	5.0	
H_2O	27.1	22.1	
Total	50.0	50.0	
5X IB2 buffer consists of: 250mM Tris-HCl (pH8.0) 75mM $(NH_4)_2SO_4$ 75mM Na_2SO_4 50mM $MgSO_4$ 5mM DTT 0.5% Triton X-100			

[0166] A typical reaction does not have a standard target concentration, but target copy per reaction may range from 10-50 at the lower end, for example, to 1E+6 copies in the upper end, for example, or more. In terms of molar concentrations, a 50 microliter assay with 10 copies of target is 3.32e-13 micromolar, where a 50 microliter assay with 50 copies of target is 1.66e-12 micromolar and a 50 microliter assay with 1e6 copies of target is 3.32e-8 micromolar.

[0167] The target sample may consist of, for example, purified DNA or RNA, that has been resuspended in dH_2O or TE, or sample that has not been purified. For example, endocervical swab clinical samples were collected, and sample was eluted and lysed from the swabs using Pierce's Lyse-N-Go PCR reagent (Cat # 78882). Lyse-N-Go is a proprietary formulation that is non-ionic detergent based. Aliquots of each eluted/lysed sample were then added directly to assays, and the results indicate no loss of assay activity. Assays have also been conducted using clinical samples that were collected in viral transport media (VTM), either M4 or M5. The samples collected in VTM were mixed with Pierce's Lyse-N-Go PCR reagent to lyse the target cells, and subsequently aliquots of these samples were added to assays without loss of activity. Finally, the assay has been conducted in the presence of various potential inhibitors, such as sand, soil, clay, urine and serum, and each of these inhibitors was well tolerated.

Example 2: Detection of DNA NEAR™ assay products by gel electrophoresis

[0168] The amplification reaction products can be visualized by gel electrophoresis. In the absence of target, the templates (with complementary or substantially complementary 3' bases) overlap by one or more bases, polymerase extends in each direction to generate the NEAR™ amplification duplex (Figure 1B); and the amplification proceeds in a similar mechanism to the NEAR™ amplification to amplify a product that is two bases shorter than the target amplified product. In the case of a 25mer assay where the templates end in A and T, the resulting background product is 23 bases. The 27mer assay also forms a 23mer background and 27mer product. Longer reaction products are also amplified. The sequence of these products is hypothesized to be due to the polymerase extension before the nicking enzyme can nick both sides of the NEAR™ amplification duplex, according to Steps 9B in Figure 1C. Figure 2 shows the NEAR™ reaction products are easily distinguished from background products by gel electrophoresis.

Example 3: Detection of RNA assay products by gel electrophoresis

[0169] The reaction of the present method can also amplify RNA targets. In this case, the target is Ebola Armored RNA, which is a ~600 base strand of RNA encapsulated by MS2 phage coat proteins to simulate a viral particle. The reaction is designed to amplify a 25-base region of the Ebola genome contained within the encapsulated RNA sequence. Reaction products run on a 20% polyacrylamide gel (Figure 3) show the amplified 25mer product along with 23mer and 20mer background products. This example demonstrates the ability of the reaction to amplify RNA released from virus-like particles.

Example 4: Detection of DNA and RNA assay products by mass spectrometry

[0170] The reaction amplification products of the present methods can also be detected by mass spectrometry using an ESI/TOF system with a front end LC. The reaction products observed are multiple charged ion species. Usually, the -3 or -4 charge state is the major peak in the spectrum (in the range of 1000-3000 AMU), depending on the length of the oligonucleotide product. The sodium adduct is usually present in the spectrum as a peak adjacent to the major peak at roughly 20-25% the intensity. The unique peaks for the positive reactions in the presence of target are visible in both Figures 4 and 5 for the DNA and RNA reactions respectively. The background products formed in these reactions are not shown in the mass range of these spectra.

Example 5: Real-time detection of the assay amplification

[0171] The amplification reaction of the present method can also be monitored, as shown in Figure 6, in real-time with SYBR II fluorescence. The fluorescence increases as SYBR II intercalates into the amplified double-stranded products. The background products also generate fluorescence at a slower rate than the true product. Optimization of amplification sequence, reaction temperature and reaction buffer conditions are necessary in order to visualize distinct separation between the positive reactions and the negative controls.

Example 6: FRET detection of real-time NEAR™ assay amplification

[0172] NEAR™ amplification can also be monitored by Fluorescence Resonance Energy Transfer (FRET), as shown in Figure 7. Amplification occurs using dual labeled templates, one on each end (5'-FAM, 3'-BHQ). Fluorescence is generated from the FAM-labeled oligonucleotide upon cleavage of the template by the nicking enzyme when it becomes double-stranded. Since fluorescence is produced by the initial nicking reaction, this detection method is extremely responsive. Since the 3' ends of the templates are blocked from extension by the quenching label, the production of background fluorescence is inhibited.

Example 7: Molecular beacon detection of real-time NEAR™ amplification

[0173] A third method of monitoring real-time amplification is using molecular beacons, as shown in Figure 8. In this case, the amplified product hybridizes to the loop region of the molecular beacon resulting in an increase in fluorescence from the separation of the fluorophore and quencher on each end of the hairpin stem. Since this interaction occurs post-amplification, it is considered pseudo-real-time and can be slightly slower in response relative to the FRET approach.

Example 8: False Positive Rate testing

[0174] This experiment was designed to probe the probability that the amplification reaction of the present method will yield a true product in the negative reaction, or a false positive. Reactions directed at specific amplification of a 25mer region specific to the *Bacillus subtilis* genome were run in the presence (n = 120) and absence (n = 320) of *Bacillus subtilis* genomic DNA. End point reactions were run on the mass spectrometer and the area under the curve (AUC) for the product mass peak in the mass spectrum was analyzed. As shown in Figure 9, the results show that none of the 320 negative reactions resulted in a false positive with AUC values equal to the water control. The true positive AUC values were at least 3 standard deviations apart from the true negatives. Overall, these results demonstrate the reproducible nature of the assays of the present methods.

[0175] The Bacillus subtilis assay was developed to target a 25 nucleotide region of the mobA-nprE gene region, with the sequence 5' - TTAACGTCTCTAATTTCAGCTTTTG - 3'. The templates used to amplify this region were, T1 5' - ATGCATGCATGAGTCACATTTAACGTCTCTA - 3', and T2 5' - ATGCATGCATGAGTCACATCAAAAGCTGAAA - 3'. The assay was carried out essentially as described in Example 1, and with the modifications here, for 4 minutes at 56°C with 10,000 copies of Bacillus subtilis genomic DNA plus 100,000 copies of Bacillus thuringiensis genomic DNA (True positives), 10,000 copies of Escherichia coli genomic DNA plus 100,000 Bacillus thuringiensis genomic DNA (True negatives) or no target (water control). Aliquots of each sample were then analyzed by electrospray ionization mass spectrometry to determine the amount of specific product made in each reaction using area under the curve (AUC) calculations.

Example 9: Beacon detection: assay reproducibility with beacon detection

[0176] The molecular beacon detection of reaction products of the present method can also be used as an endpoint reading. As shown in Figure 10, the ratio of reaction products can be manipulated by varying the input ratio of the forward

and reverse templates. Skewing the templates to favor one of the reaction products allows the single-stranded product to be available for hybridization to a molecular beacon. The open beacon generates a fluorescent signal. This detection method is extremely reproducible. In this study, two operators performed replicates of the same assay on two different days. The results of this study demonstrate the reproducibility of the assay from one day to the next as well as reproducibility between operators.

Example 10: Assay sensitivity with beacon detection

[0177] The sensitivity of the assay with beacon read-out was tested using a dilution of *Francisella tularensis* genomic DNA. As shown in Figure 11, as few as 50 copies were detected above the no target control.

Example 11: Concentration of amplified products for DNA amplification

[0178] The sensitivity of the assay has also been studied using mass spectrometry detection of the reaction products. Figure 12 shows signal above the no target control down to 100 copies. The data from this study was used to correlate the input copy number to the final amount of amplified product. In this study, the AUC values of the mass spec product peaks were fit to a standard curve to give the estimated final concentration of amplified product for the assay. The amount of amplified product ranges from approximately 250nM to almost 1μM for 1E+2 and 1E+5 copies respectively. This product amount results in a 1E+8 to 7E+10-fold amplification. These reactions were performed without the hot-start conditions, in fact hot-start conditions have been shown to dramatically increase the amount of product amplified, so a further increase in amplification is achieved. The zero copy amplification reaction has a positive final concentration due to the y-intercept value in the standard curve equation.

Example 12: Concentration of amplified products for RNA assay

[0179] A similar study was performed on the amplification of RNA using the present method. A dilution of RNA targets were amplified by the assay of the present method. Products were run on the mass spec and the AUC values of the product peaks were analyzed against a standard curve to determine the concentration of the final product, as shown in Figure 13. A 12 minute amplification starting with 30 and 30,000 copies of initial target results in a 3E+9 to 1E+7 -fold amplification respectively. The lower extent of amplification compared to the DNA amplification could be due to the less efficient reverse transcriptase ability of the polymerase compared to its replication abilities. Also, the RNA:DNA hybrid formed upon the extension of the reverse template is a stronger interaction compared to a normal DNA:DNA hybrid and will have less breathing to allow for the forward or another reverse template to displace one strand. However, amplification products from the RNA reaction were detected down to <100 copies.

Example 13: NEAR™ reaction specificity for DNA

[0180] Since the reaction products are usually between 20 and 30 bases in length, the question arises as to whether or not these short amplification assays can be specific enough to target a single sequence region with other near neighbor genomes present. The reaction was tested for its specificity by running the amplification reaction in the presence and absence of varying amounts of the near neighbor genomic DNA (Figure 14). In this case, the assay detects a specific sequence in the pXO2 plasmid of *Bacillus anthracis* and the near neighbor genome is *Bacillus thuringiensis* (kurstaki). The reactions were analyzed by the AUC values for the product peaks. The figure below demonstrates that in the absence of the correct target (*Bacillus anthracis*), there is no true product amplified (the levels are so low that they are not visible on the scale of the graph). The amount of amplification of the positive reactions is consistent, with larger error bars for the 0 and 5E+5 copies of *Bacillus thuringiensis* (5E+4 copies of *Bacillus anthracis*) due to a single lower value for one of the triplicate runs. Overall the experiment demonstrates that the reaction is very specific to the target sequence when the assay is designed within a unique region of the genome.

Example 14: Interferent testing

[0181] A panel of interferents was tested to monitor the effect of each on amplification. Figure 15 demonstrates the robust nature of the assay of the present methodin the presence of interferents. Some interferents that are known to inhibit PCR, such as humic acid, did not appear to inhibit the assay, though the amount of each interferent is unknown. From statistical analysis only interferent B, C, and E were statistically different from the control assay x. In the B, C, and E cases, the difference resulted in increased product amplification.

Example 15: Multiplexing of two sequences with DNA assays

[0182] A DNA duplex was designed for capillary electrophoresis (CE) detection. Amplification products were 25 bases (*Bacillus anthracis* assay, *Ba*) and 27 bases (*Bacillus subtilis* assay, *Bs*) in length with background production of a 23mer. The reaction was run for 10 minutes in the presence or absence of 5E+5 copies of the respective genomic DNA target. The samples were run on a 20% polyacrylamide gel to visualize the reaction products. Figure 16 indicates the presence of positive product amplification when *Bacillus subtilis* only is present as well as when both *Bacillus subtilis* and *Bacillus anthracis* are present.

Example 16: DNA assay duplex specificity

[0183] The DNA duplex reaction with *Bacillus subtilis* (*Bs*) and *Bacillus anthracis* (*Ba*) was shown to be specific to the respective genomes. The assays were run in the presence of the near neighbor, *Bacillus thuringiensis*, as shown in Figure 17. In the negative reaction where both template sets are present as well as the *Bacillus thuringiensis* genomic DNA, there is no product band in the 25 or 27mer region. Product bands appear only when the specific genomic target is present, which demonstrates the specificity of the duplex reaction.

Example 17: Multiplexing with RNA assays

[0184] An MS2 assay that amplifies a 27mer product and an Ebola assay that amplifies a 25mer product was developed and multiplexed so that all templates are present in each assay and amplification of products is dependent on the target present. This combination of templates forms background products that are 23 bases and 20 bases in length. The gel shown in Figure 18 demonstrates the ability for the reaction of the present method to amplify multiple RNA targets in a single reaction.

Example 18: Amplification from lysed spores

[0185] Amplification was performed on semi-processed samples to determine whether it is possible to amplify DNA released from spores through lysis. The negative control reaction contained DNase-treated spores, unlysed, so no DNA should be present to amplify. The positive control reaction contained purified genomic DNA at concentrations around the amount of DNA estimated to be released through lysis. Results in Figure 19 show that amplification with unlysed DNase-treated spores results in no product amplification as expected, whereas the three samples lysed before amplification resulted in product amounts in the range of the theoretical amounts.

Example 19: Capture and Extension

[0186] The reaction products of the present method can also be detected on a solid surface. A capture probe attached at the 5' end to the surface through a biotin/streptavidin attachment can bind to the reaction products from which polymerase extends to form a stable duplex that SYBR and any intercalating dye can detect. The capture probe is designed to favor extension through binding to the true product over background products because the 3' base of the capture probe is complementary to the middle spacer base in the product which is not present in either of the templates or the background products. Figure 20 demonstrates the increased fluorescence of the products in the presence of the capture probe and polymerase over the average binding (same reaction in the absence of polymerase, to preclude extension of the capture probe) and the no target control where only background products are amplified, but cannot form a stable duplex with the capture probe for polymerase to extend.

Example 20: Surface NEAR™ FRET DNA Assay

[0187] The reaction of the present method can also be performed with the templates immobilized on the surface. The templates for FRET detection of surface amplification usually have three modifications: one 5' biotin with a TEG spacer, one FAM fluorophore internal to the biotin, and a quencher on the 3' end which serves to block background amplification as well as to quench the FAM fluorophore. The template is immobilized on the surface through biotin/streptavidin attachment. Figure 21 demonstrates that with both templates immobilized along with additional mixing, the reaction proceeds at a much slower rate than the solution amplification rate (amplification in 16 minutes for 1E+6 copies of genomic DNA). When a single template is immobilized on the surface and the other template is free in solution, the amplification reaction is increased to 10 minute detection for 1E+6 copies of genomic DNA. Fluorescence from background products is observed ~3.5 minutes after the product signal, similar to what is observed for solution phase kinetics, but slowed considerably.

Example 21: Healthcare Example

Chlamydia trachomatis (Ct) Assay

5 **[0188]** An assay of the present method was performed to detect the presence of a Chlamydia trachomatis (Ct) target sequence. A 2-fold dilution series of synthetic DNA containing the target sequence for the Ct P2_2 assay was used to determine the limit of detection of the assay. The reaction was carried out essentially as described in Example 1, with some modifications as described in this example. The dilution series started with 10,000 copies of target DNA, and proceeded to less than 1 copy per reaction. A 'no target' control sample was also included in this experiment. Reactions 10 were performed on a 96-well microtiter plate in 50 microliter volumes in the following buffer: 50 mM Tris-HCl, pH 8.0, 30 mM NaCl, 15 mM $(NH_4)_2(SO_2)$, 15 mM Mg_2SO_4, 1 mM DTT, 0.1% Triton X-100 with 0.3 mM dNTPs, 19.2 units of Bst DNA polymerase and 15 units of Nt.BstNBI nicking enzyme. Templates were added at a ratio of 200 nM :100 nM (Template 1:Template 2). Reactions were performed as follows: On plate 1, 5 microliters of template mix was added to each well in a pre-amplification room, and sealed. On plate 2, 40 microliters of master mix was added to each well in a 15 pre-amplification room, and sealed. The master mix consisted of dH_2O plus all assay components listed above, except templates. The two plates were then transferred to a post-amplification room where 5 microliters of target was added to each well of plate 1 (excluding the 'no target' control wells). The two plates were then transferred to thermal cyclers pre-heated to 56°C for 2 - 3 minute pre-incubations at 56°C. The contents of plate 2 were then transferred to plate 1 which was then incubated for 5 minutes at 56°C (amplification step). Following this incubation, the reactions were stopped by 20 inactivating the enzymes at 80°C for 2 minutes. Subsequently, a molecular beacon specific for the amplified Ct P2_2 product was added to a final concentration of 300 nM and fluorescence was detected at 56°C. All samples were performed in triplicate, with error bars showing standard deviations.

[0189] The Ct P2_2 assay was performed using two templates, template 1 (5'-ATGCATGCATGAGTCACATAGGCT-TATGGAG-3') and template 2 (5'-ATGCATGCATGAGTCACATTTATACCGCTTA-3') at a 200 nM:100 nM final template 25 concentration. The molecular beacon used for fluorescence detection, MB 5.18, contained a 5'-FAM fluorophore and 3'-BHQ1 quencher, with the following sequence: 5'-ctggcTACCGCTTAACTCCATAAgccag-3'.

[0190] The results are shown in Figure 22, and show that the assay can efficiently detect less than 10 copies of target in a sample. Figure 22B shows that even about 1-2 copies can be detected, but because of the dilution experiment, some wells may, statistically, not have any target DNA (compare Fig. 22b, bars 1.2 a, b, and c).

30 **[0191]** The target sequence for the Ct P2_2 assay is 5'AGGCTTATGGAGTTAAGCGGTATAA - 3'. Clinical samples, such as those collected on endocervical or vaginal swabs, or those collected on swabs and then transferred to viral transport media such as M4 or M5 can be prepared for use in anassay as follows. Each swab is placed into a 1.5 milliliter or 2.0 milliliter eppendorf tube containing 300 microliters to 1 milliliter of Pierce's Lyse-N-Go PCR reagent (Cat # 78882). The mixture is allowed to incubate at room temperature for 5 - 10 minutes, with occasional mixing. An aliquot of the 35 eluted and lysed sample is then added directly to an assay. For samples present in viral transport media, an aliquot of the sample can be transferred to an eppendorf tube containing an equal or greater volume of Pierce's Lyse-N-Go PCR reagent (at a sample:Lyse-N-Go ratio of 1:1, 1:2, 1:10, 1:20, etc....) and allowed to incubate at room temperature for 5 - 10 minutes, with occasional mixing. An aliquot of the eluted and lysed sample is then added directly to an assay.

40 **Example 22** Food Safety Applications

Listeria monocytogenes assay

[0192] To demonstrate the effectiveness of the assay of the present method for the specific detection of a food 45 pathogen, assays were conducted on *Listeria monocytogenes*, one of the most significant threats to food safety from ready-to-eat food products. The assays were performed essentially as described in Example 1, with modifications described in this Example. *L. monocytogenes* strain EGD-e genomic DNA was assayed with increasing amounts of genomic DNA from the closely related non-pathogenic species *L. innocua* strain Clip11262. As shown in Figure 23, Negative control reactions with no DNA present showed only background levels of fluorescence, and the increasing amounts of 50 *L. innocua* DNA up to 1 million genome equivalents per 50 microliter reaction showed no significant increase in the background fluorescence. However, the addition of 1,000 genome equivalents of *L. monocytogenes* was easily detected with a substantial increase in fluorescence, and was unaffected by the presence of the *L. innocua*, even when the non-pathogenic *L. innocua* was present in 1000-fold excess, which was 1 million genome equivalents per 50 microliter reaction. Each reaction consisted of: 46 mM Tris buffer pH 8.5; 50 mM NaCl; 10 mM KCl; 10 mM $(NH_4)_2SO_4$; 5 mM 55 $MgCl_2$; 10 mM $MgSO_4$; 0.5 mM dithiothreitol; 0.1% Triton X-100; 0.01 mM EDTA; 0.3 mM each dATP, dCTP, dGTP, and dTTP; 19.2 units *Bst* DNA polymerase from New England Biolabs, Inc.; 15 units Nt.BstNBI nicking endonuclease from New England Biolabs, Inc.; 200 nM of the first oligonucleotide; and 2 micromolar of the second oligonucleotide. The oligonucleotides and *Listeria* genomic DNA were incubated separately from the enzyme buffer mixture at 56 °C,

and then 5 microliters of this mixture was added to 45 microliters of enzyme buffer mixture. The reaction was incubated at 56 °C for 10 minutes, and then 80 °C for two minutes. After this, 3.2 microliters of a 5 μM solution of a Molecular Beacon was added to each reaction. The sequence of the Molecular Beacon was specific for the amplified *L. monocytogenes* sequence with a fluorophore and quencher on the 5' and 3' ends, respectively. Following the addition of the Molecular Beacons, the reactions were incubated at 56 °C for one minute, and then fluorescence measurements were made. Each assay condition was tested in duplicate, and the average fluorescence values are shown. The target sequence for the Listeria monocytogenes assay is 5' - AAAGCAAGAGAAAGTTATCGTGTAT - 3'. The template sequences are as follow: T1 5' - ATGCATGCATGAGTCACATAAAGCAAGAGAA - 3'and T2 5' - ATGCATGCATGAGTCACATATACACGATAAC - 3'.

Example 23: Viral RNA Example

[0193] A 10-fold dilution series of purified viral RNA from a viral positive clinical sample was used to determine the limit of detection of the assay. The viral RNA was purified using a commercially available viral RNA purification kit. A 'no target' negative control sample was included. Reactions were performed on 96-well microtiter plates in 50 microliter volumes in the following buffer: 50 mM Tris-HCl, pH 8.0, 30 mM NaCl, 15 mM $(NH_4)_2(SO_2)$, 10 mM Mg_2SO_4, 1 mM DTT, 0.1% Triton X-100 with 0.1 mM dNTPs, 19.2 units of Bst DNA polymerase, 7.5 units of Nt.BstNBI nicking enzyme and 4 units of OmniScript reverse transcriptase. Templates were added at a ratio of 400 nM : 20 nM (Template 1: Template 2). Reactions were performed as follows: On plate 1, 5 microliters of template mix was added to each well in a pre-amplification room, and sealed. On plate 2, 40 microliters of master mix was added to each well in a pre-amplification room, and sealed. The master mix consisted of water plus all assay components listed above, except templates. The two plates were then transferred to a post-amplification room where 5 microliters of target was added to each well of plate 1 (excluding the 'no target' control wells). The two plates were then transferred to thermal cyclers pre-heated to 56°C for 2 - 3 minute pre-incubations at 56°C. The contents of plate 2 were then transferred to plate 1 which was then incubated for 5 minutes at 56°C (amplification step). Following this incubation, the reactions were stopped by inactivating the enzymes at 80°C for 2 minutes. Subsequently, molecular beacon specific for the amplified product was added to a final concentration of 300 nM and fluorescence was detected at 56°C. All samples were performed in triplicate, with error bars showing standard deviations. Results are shown in Figure 24.

[0194] The viral RNA assay was performed using two templates (template 1: 31 nucleotides long, and template 2: 31 nucleotides long) at a 400 nM:20 nM final template concentration. The molecular beacon used for fluorescence detection (MB), contained a 5'-FAM fluorophore and 3'-BHQ1 quencher, with a 29 nucleotide long sequence. The length of the target sequence was 26 nucleotides.

Example 24: Agriculture Application: Detection of Genetically Modified Traits in Crops Assay Sample Preparation for Genetically Modified (GMO) and Conventional (non-GMO) Maize:

[0195] The assay of the present methods may be used to detect genetically modified organisms (GMO) in agricultural applications. Theassay was used to detect the presence of the *bar* gene, inserted into the maize genome, in a background of unmodified maize DNA. The *bar* gene confers resistance to the broad-spectrum herbicide glufosinate. The assays were conducted essentially as described in Example 1, with modifications as described herein. Genetically modified and conventional (unmodified) maize seeds were ground to an appropriate level of coarseness, and nucleic acids were extracted using a standard buffer. The extracted material was purified using a size-exclusion column according to the manufacturer's instructions. Purified nucleic acids were combined to yield a final concentration of 5% *bar*-modified maize in a conventional background (*e.g.*, 5 microliters of *bar* maize DNA extract combined with 95 microliters conventional maize DNA extract), or used unmixed in the case of 100% conventional maize. The oligonucleotide sequences used to detect the bar gene are listed below.
Template 1: ATGCATGCATGAGTCACATCATCGTCAACCA
Template 2: ATGCATGCATGAGTCACATTGTCTCGATGTA

[0196] The templates were designed to produce the following products:

Product 1: CATCGTCAACCACTACATCGAGACA
Product 2: TGTCTCGATGTAGTGGTTGACGATG

[0197] The assay reagents used were: 9.6 units of Bst. Polymerase (NEB), 15 units of N.BstNBI nicking enzyme (NEB), 5 microliters Thermopol I Buffer (NEB), 2.5 microliters NEB Buffer 3, 12 mM $MgSO_4$, 0.3 mM dNTPs, 2.5% DMSO (dimethyl sulfoxide), 5 microliters sample, templates and water. The oligonucleotides were present at initial concentrations of 10 nM (Template 1) and 100 nM (Template 2). Water was used to adjust the final volume to 50 microliters, and a 10 minute assay was performed at 56° C, followed by a 2 minute incubation at 94° C to inactivate the enzymes, followed

by detection at 56° C with a specific molecular beacon at a final concentration of 300 nM. The sequence of this molecular beacon is:

5' FAM-CCTCGCCGTCAACCACTACATCGAGCGAGG-BHQ1-3'.
The results are shown in Figure 25.

Example 25: Detection of MicroRNA (miRNA)

Assay Sample Preparation for microRNAs from MDA-MB-231 Human Breast Cancer Cells:

[0198] MDA-MB-231 Human breast cancer cells (ATCC number HTB-26) are known to express elevated levels of microRNA-21 (Iorio, M.V. et al., 2005. MicroRNA gene expression deregulation in human breast cancer. Cancer Res. 65:7065-7070). An assay for miR-21 was developed that detects the mature microRNA-21 sequence:

5'UAGCUUAUCAGACUGAUGUUGA3'

[0199] The template sequences used were (nicking enzyme sequences are underlined):

Template 1: ATGCATGCATGAGTCACATTAGCTTATCA
Template 2: ATGCATGCATGAGTCACATTCAACATCAG

[0200] The templates were designed to produce the following products:

Product 1: TAGCTTATCAGACTGATGTTGA
Product 2: TCAACATCAGTCTGATAAGCTA

[0201] The assay was conducted essentially as described in Example 1, with modifications described herein. To obtain RNA, MDA-MB-231 cells were propagated and sub-cultured, using standard methods familiar to those skilled in the art, in Dulbecco's Modified Eagle's Medium (Invitrogen) supplemented with 10% fetal bovine serum, glucose and antibiotics. Prior to reaching confluency, cells were removed from the plate by treatment with trypsin, and subsequently washed in phosphate buffered saline prior to freezing at -80° C. Cells were later defrosted and a portion used for RNA isolation with TRI Reagent (Molecular Research Center, Inc.) according to the manufacturer's instructions. Purified RNA was quantified using UV absorbance at 260 nm.

[0202] According to the Molecular Research Center TRI Reagent manual, 1 ng of purified RNA corresponds to approximately 100 cells of starting material. Various amounts of purified RNA were used in an assay comprised of the following reagents: 50 mM Tris-HCl, pH 8.0, 30 mM $(NH_4)_2SO_4$, 30 mM Na_2SO_4, 1 mM DTT, 0.1% Triton X-100, 10 mM $MgSO_4$, 0.1 mM dNTPs, 19.2 units of Bst. Polymerase (New England Biolabs), 7.5 units of N.BstNBI nicking enzyme (New England Biolabs), 7.4 units Omniscript Reverse Transcriptase (Qiagen), two oligonucleotides at 100 nM each, sample and water. Water was used to adjust the final volume to 50 microliters, and a 20 minute assay was performed at 56° C, followed by a 2 minute incubation at 94° C to inactivate enzymes. The product was measured using electrospray ionization mass spectrometry, and product amounts were quantified by calculating the area under the curve. The results of the assay are shown in Figure 26.

Example 26: Detection of a Genomic DNA Target

[0203] An assay of the present method was performed essentially as described in Example 1, using oligo templates designed to bind to a genomic target. Dilution experiments were conducted to determine the lower limit of detection. As shown in Figure 27, there was consistent detection at 50 genome copies. When the diluted sample contained 10 genomic copies, there was detection, however, statistically, the detection was not as consistent.

[0204] Example 26 (Figure 27) depicts a Neisseria gonorrhoeae assay. The assay targets the pilQ gene, specifically the sequence 5' - ACTCTACCAACACGGAACTCAAAAA - 3'. The template sequences used to amplify this target were: T1 5' - ATGCATGCATGAGTCACATTTTTTGAGTTCC - 3', and T2 5' - ATGCATGCATGAGTCACATACTCTACCAACA - 3'. The assay was carried out essentially as described in Example 1, with the modifications herein. Briefly, the assay was performed for 5 minutes at 56°C followed by a heat inactivation step at 80°C for 2 minutes to stop the reaction. End point detection of amplified specific product was performed using 300 nanomolar of a molecular beacon containing a 5'-fluorophore and 3'-quencher that was specific to the amplified specific following a 1 minute incubation at 56°C. The molecular beacon sequence was: 5' - CGCATGGAGTTCCGTGTTGGTAGACATGCG - 3'.

[0205] **Example 27:** Calculation of Specific Product Generated in a B. subtilisAssay Anassay of the present method

was performed essentially as described in Example 1, using oligo templates designed to bind to a Bacillis subtilis target sequence, the target was the ppsA gene:

Target sequence (25mer) 5' - CCAAGCTCAAAAAAGGAATCGTGAA - 3'
T1 5' - ATGCATGCATGAGTCACATCCAAGCTCAAAA - 3'
T2 5' - ATGCATGCATGAGTCACATTTCACGATTCCT - 3'

[0206] As shown in Figure 28, the linear regression showed an excellent correlation between the amount of the reference oligo added to a sample and area under the curve (AUC). This equation was used to determine the amount of specific product generated when 50 or 500 copies of genomic DNA target were added to a reaction. The reaction was performed for 5 minutes. The fold amplification was calculated and is presented in the Table below.

Table 5

Specific product 1944 yields (x=y-b/m)				
Sample	**AUC signal**	**Product (nM)**	**Product (50 microliter reaction, in pmoles)**	**Fold amplification**
50-1	1394	2851	0.1426	1.72E+09
50-2	1495	3049	0.1525	1.84E+09
50-3	1175	2421	0.1211	1.46E+09
50-4	1072	2219	0.1110	1.34E+09
500-1	1799	3645	0.1823	2.20E+08
500-2	1837	3720	0.1860	2.24E+08
500-3	1472	3004	0.1502	1.81E+08
500-4	1438	2937	0.1469	1.77E+08

[0207] Calculations were based on the following: B. subtilis genome = 4214814 nucleotides, molecular weight (g/mole) of 2781777240. Avogadro's number (molecules/mole) = 6.02E+23. For 50 genome copies in moles, this results in 8.30E-23, for 500 genome copies in moles, this results in 8.30E-22.

Example 28: Effect of Different Spacer Lengths

[0208] A series of *Chlamydia trachomatis* (Ct) assays was performed essentially as describe in Examples 1 and 21, using various templates as shown in Figures 29 and 30. Figure 29 shows the results of the reaction, Figure 30 provides more detail as to the template design. The reaction was conducted for 10 minutes using either 0 or 100 copies of target. A series of oligonucleotide templates was prepared, with spacer region lengths (number of nucleotides on the target sequence between the binding sites of the oligo templates, if the templates were bound) ranging from 1 to 11. Optimal spacer lengths for this experiment were 1, 2, 3, or 4.

[0209] A similar set of experiments was conducted for a viral RNA target, following essentially the same methods as those described in Example 23m using spacer lengths of 2, 5, 6, 7, and 8. As determined by mass spectrometry, optimal specific product detection was found using spacer lengths of 2 and 5, and no specific product was detected in this assay where the spacer length was 6 or greater and the reaction was run for 20 minutes.

[0210] Similar experiments were also conducted with other targets. For some targets, such as miR-21, when no spacer nucleotides were included in the template design, product was detected whether or not a target sequence was present in the reaction. Product was detected whether or not target DNA was present in the assay, indicating that the template set was producing the specific product without a need for the target being present. In other experiments, a spacer region of 0 nucleotides did result in specific product. Therefore, in designing templates for the assays discussed herein, more than one set of templates should be prepared, to determine the length of the spacer region that is optimal to produce specific product from a particular target.

Example 29: Effect of the Stabilizing Regions

[0211] A set of *Chlamydia trachomatis* (Ct) assays was performed essentially as described in Examples 1 and 21. Templates were prepared that either included, or did not include, the stabilizing region (5'ATGCATGCAT). The reaction was performed for 10 minutes, with either 0 or 100 copies of target DNA. Analysis was performed using real-time

SybrGreen fluorescence detection. As shown in Figure 31, the samples containing templates without stabilizing regions showed no amplification. In another set of assays, using viral RNA, either 0 or 1000 copies of target was included in the assay. The samples containing templates without stabilizing regions showed no amplification, while those with stabilizing regions showed rapid amplification.

Example 30: Effect of Mg^{+2} Concentration

[0212] A set of *Chlamydia trachomatis* (Ct) assays was performed essentially as described in Examples 1 and 21. The assays were conducted using varying concentrations of Mg^{+2}. As shown in Figure 32, for this set of assays, a complete loss of activity was found when 6 mM Mg^{+2} was present, and a significant drop in activity was found when 9 mM Mg^{+2} was present. At concentrations from 12 mM to 21 mM Mg^{+2}, the assay performed optimally.

Example 31: Examples of other Template/Target Combinations

[0213] The present methods are not limited to the specific templates and targets provided in the present embodiments and examples. Other targets and templates may be used to perform the isothermal amplification methods discussed herein. Examples of other targets and templates include, but are not limited to, those presented in Figure 34. Those of ordinary skill in the art recognize that other templates may be designed for the targets presented in the Figure, related target sequences to those presented in the Figure may be used in the reaction, and target sequences not included in the Figure are within the scope of the present methods.

[0214] The entirety of each patent, patent application, publication and document referenced herein hereby is incorporated by reference. Citation of the above patents, patent applications, publications and documents is not an admission that any of the foregoing is pertinent prior art, nor does it constitute any admission as to the contents or date of these publications or documents.

[0215] Singular forms "a", "an", and "the" include plural reference unless the context clearly dictates otherwise. Thus, for example, reference to "a subset" includes a plurality of such subsets, reference to "a nucleic acid" includes one or more nucleic acids and equivalents thereof known to those skilled in the art, and so forth. The term "or" is not meant to be exclusive to one or the terms it designates. For example, as it is used in a phrase of the structure "A or B" may denote A alone, B alone, or both A and B.

[0216] Unless defined otherwise, all technical and scientific terms used herein have the same meanings as commonly understood by one of ordinary skill in the art to which this invention belongs. Although any methods and systems similar or equivalent to those described herein can be used in the practice or testing of the present invention, the methods, devices, and materials are now described. All publications mentioned herein are incorporated herein by reference for the purpose of describing and disclosing the processes, systems, and methodologies that are reported in the publications which might be used in connection with the invention. Nothing herein is to be construed as an admission that the invention is not entitled to antedate such disclosure by virtue of prior invention.

[0217] Modifications may be made to the foregoing without departing from the basic aspects of the invention. Although the invention has been described in substantial detail with reference to one or more specific embodiments, those of ordinary skill in the art will recognize that changes may be made to the embodiments specifically disclosed in this application, and yet these modifications and improvements are within the scope and spirit of the invention. The invention illustratively described herein suitably may be practiced in the absence of any element(s) not specifically disclosed herein. Thus, for example, in each instance herein any of the terms "comprising", "consisting essentially of", and "consisting of" may be replaced with either of the other two terms. Thus, the terms and expressions which have been employed are used as terms of description and not of limitation, equivalents of the features shown and described, or portions thereof, are not excluded, and it is recognized that various modifications are possible within the scope of the invention. Embodiments of the invention are set forth in the following claims.

[0218] Paragraphs of the invention:

1. A method for nucleotide sequence amplification, which comprises:

combining a target nucleic acid having a target nucleotide sequence with (i) a polymerase, (ii) a first template nucleic acid that hybridizes to the a first strand of the target nucleotide sequence, and (iii) a second template nucleic acid that hybridizes to the complement of the first strand of the target nucleotide sequence,
in an amplification reaction,
under conditions in which the polymerase extends the template nucleic acids,
thereby generating extended template nucleic acid amplicons; wherein:

the target nucleotide sequence is between 20 and 40 nucleotides in length;

the target nucleotide sequence is amplified 1E+6-fold or more in about ten minutes; and
the foregoing steps are conducted under substantially isothermal conditions.

2. A method for nucleotide sequence amplification, which comprises:

combining a target nucleic acid having a target nucleotide sequence with (i) a polymerase, (ii) a first template nucleic acid that hybridizes to the a first strand of the target nucleotide sequence, and (iii) a second template nucleic acid that hybridizes to the complement of the first strand of the target nucleotide sequence,
in an amplification reaction,
under conditions in which the polymerase extends the template nucleic acids,
thereby generating extended template nucleic acid amplicons; wherein:

the target nucleotide sequence is between 20 and 40 nucleotides in length;
the first template comprises a nucleic acid sequence comprising a first template recognition region at the 3' end that is complementary or substantially complementary to the 3' end of the first strand of the target nucleotide sequence;
the second template comprises a nucleotide sequence comprising a second template recognition region at the 3' end that is complementary or substantially complementary to the 3' end of the complement of the first strand of the target nucleotide sequence;
the target nucleotide sequence comprises from 1 to 5 nucleotides more than the sum of the nucleotides of the first template recognition region and the second template recognition region;
the target nucleotide sequence is amplified 1E+6-fold or more in about ten minutes; and the foregoing steps are conducted under substantially isothermal conditions.

3. A method for nucleotide sequence amplification, which comprises:

combining a target nucleic acid having a target nucleotide sequence with (i) a polymerase, (ii) a first template nucleic acid that hybridizes to the a first strand of the target nucleotide sequence, and (iii) a second template nucleic acid that hybridizes to the complement of the first strand of the target nucleotide sequence,
in an amplification reaction,
under conditions in which the polymerase extends the template nucleic acids,
thereby generating extended template nucleic acid amplicons; wherein:

the first template comprises a nucleic acid sequence comprising a first template recognition region at the 3' end that is complementary or substantially complementary to the 3' end of the first strand of the target nucleotide sequence,
wherein the recognition region is 8-15 nucleotides long;
the second template comprises a nucleotide sequence comprising a second template recognition region at the 3' end that is complementary or substantially complementary to the 3' end of the complement of the first strand of the target nucleotide sequence, wherein the recognition region is 8-15 nucleotides long;
the target nucleotide sequence is amplified 1E+6-fold or more in about ten minutes; and the foregoing steps are conducted under substantially isothermal conditions.

4. The method of paragraph 1, wherein
the first template comprises a nucleic acid sequence comprising a first template recognition region at the 3' end that is complementary or substantially complementary to the 3' end of the first strand of the target nucleotide sequence; and
the second template comprises a nucleotide sequence comprising a second template recognition region at the 3' end that is complementary or substantially complementary to the 3' end of the complement of the first strand of the target nucleotide sequence.
5. The method of paragraph 3, wherein the target nucleotide sequence comprises from 1 to 5 nucleotides more than the sum of the nucleotides of the first template recognition region and the second template recognition region.
6. The method of any of paragraphs 2, 3, 4, or 5, wherein the first template and second templates comprise nicking enzyme binding sites and nicking sites upstream of the recognition regions, and the amplification reaction further comprises one or more nicking enzymes that are capable of nicking at the nicking site of said forward and said reverse templates, wherein either one nicking enzyme is capable of nicking both of said templates, or each template is capable of being nicked by at least one of the nicking enzymes, and wherein said one or more nicking enzymes do not nick within said target sequence.

7. The method of paragraph 6, wherein the target nucleotide sequence comprises 1 nucleotide more than the sum of the nucleotides of the first template recognition region and the second template recognition region.
8. The method of paragraph 6, wherein the target nucleotide sequence comprises 2 nucleotides more than the sum of the nucleotides of the first template recognition region and the second template recognition region.
9. The method of paragraph 6, wherein the target nucleotide sequence comprises 3 nucleotides more than the sum of the nucleotides of the first template recognition region and the second template recognition region.
10. The method of any of paragraphs 1-9, wherein the target nucleic acid is double stranded or single stranded.
11. The method of paragraph 10, wherein the target nucleic acid is double-stranded DNA.
12. The method of paragraph 10, wherein the target nucleic acid is single-stranded DNA.
13. The method of paragraph 10, wherein the target nucleic acid is RNA
14. The method of paragraph 11, wherein the target nucleic acid is selected from the group consisting of genomic DNA, plasmid DNA, viral DNA, mitochondrial DNA, and synthetic double-stranded DNA.
15. The method of paragraph 12, wherein the target nucleic acid is selected from the group consisting of viral DNA, cDNA, and synthetic single-stranded DNA.
16. The method of paragraph 13, wherein the target nucleic acid is selected from the group consisting of messenger RNA, viral RNA, ribosomal RNA, transfer RNA, micro RNA, micro RNA precursor, and synthetic RNA.
17. The method of any of paragraphs 1-16, wherein said DNA polymerase is a thermophilic polymerase.
18. The method of any of paragraphs 1-16, wherein said polymerase is selected from the group consisting of Bst (large fragment), 9°N, $Vent_R$® (exo-) DNA Polymerase, Therminator, and Therminator II.
19. The method of any of paragraphs 1-19, wherein said polymerase is Bst (large fragment).
20. The method of any of paragraphs 6-12, wherein the first and second templates comprise nicking enzyme binding sites recognized by the same nicking enzyme and said first and said second nicking enzyme are the same.
21. The method of paragraph 19, wherein said nicking enzymes are selected from the group consisting of Nt.BspQI, Nb.BbvCi, Nb.BsmI, Nb.BsrDI, Nb.BtsI, Nt.AlwI, Nt.BbvCI, Nt.BstNBI, Nt.CviPII, Nb.Bpu10I, and NtBpu10I.
22. The method of any of paragraphs 1-20, wherein the portion of the nucleic acid sequence of the first strand that is complementary or substantially complementary to the first strand of the target nucleotide sequence is 8-15 nucleotides in length and wherein the portion of the second strand that is complementary or substantially complementary to the target nucleotide sequence is 8-15 nucleotides in length.
23. The method of any of paragraphs 1-22, wherein the first template is provided at the same concentration as the second template.
24. The method of any of paragraphs 1-22, wherein one of the first or second templates is provided at a ratio to the other template at the range of ratios of 1:100 to 100:1
25. The method of any of paragraphs 1-25, further comprising a second polymerase.
26. The method of paragraph 25, wherein at least one of the first or second polymerases comprises reverse transcriptase activity.
27. The method of any of paragraphs 1-26, wherein the amplification is conducted between 54°C and 60°C.
28. The method of any of paragraphs 1-26, wherein the amplification is conducted between 56°C and 58°C.
29. The method of any of paragraphs 1-28, wherein the amplification reaction is held at a constant temperature for 1 to 10 minutes.
30. The method of any of paragraphs 1-28, wherein the amplification reaction is held at a constant temperature for 1 to 20 minutes.
31. The method of any of paragraphs 1-30, further comprising detecting the amplification product.
32. The method of paragraph 31, wherein said amplification product is detected by detection method selected from the group consisting of gel electrophoresis, mass spectrometry, SYBR I fluorescence, SYBR II fluorescence, SYBR Gold, Pico Green, TOTO-3, intercalating dye detection, fluorescence resonance energy transfer (FRET), molecular beacon detection, surface capture, capillary electrophoresis, incorporation of labeled nucleotides to allow detection by capture, fluorescence polarization, and lateral flow capture.
33. The method of any of paragraphs 1-32, wherein at least two target sequences are capable of being amplified.
34. The method of any of paragraphs 1-33, wherein said amplification products are detected on a solid surface.
35. The method of any of paragraphs 1-34, wherein at least one capture probe is immobilized on a solid surface.
36. The method of any of paragraphs 1-35, wherein at least one of said templates comprises a spacer, blocking group, or a modified nucleotide.
37. The method of any of paragraphs 1-36, wherein the target nucleotide sequence is amplified 1E+6-fold or more in about five minutes.
38. The method of any of paragraphs 1-36, wherein the target nucleotide sequence is amplified 1E+6-fold or more in about 2.5 minutes.
39. The method of any of paragraphs 1-36, wherein the target nucleotide sequence is amplified 1E+7-fold or more in about five minutes.

40. The method of any of paragraphs 1-36, wherein the target nucleotide sequence is amplified 1E+8-fold or more in about five minutes.
41. The method of any of paragraphs 1-36, wherein the target nucleotide sequence is amplified 1E+9-fold or more in about five minutes.
42. A method for amplifying a double-stranded nucleic acid target sequence, comprising

a) contacting a target DNA molecule comprising a double-stranded target sequence, having a sense strand and an antisense strand, with a forward template and a reverse template, wherein

i) said forward template comprises a nucleic acid sequence comprising a recognition region at the 3' end that is complementary to the 3' end of the target sequence antisense strand; a nicking enzyme binding site and a nicking site upstream of said recognition region and a stabilizing region upstream of said nicking site, wherein the portion of the nucleic acid sequence that is complementary to the 3' end of the target antisense strand is 8-15 nucleotides in length;
ii) said reverse template comprises a nucleotide sequence comprising recognition region at the 3' end that is complementary to the 3' end of the target sequence sense strand, a nicking enzyme binding site and a nicking site upstream of said recognition region, and a stabilizing region upstream of said nicking site, wherein the portion of the nucleic acid sequence that is complementary to the 3' end of the target antisense strand is 8-15 nucleotides in length;

b) providing a first nicking enzyme that is capable of nicking upstream, downstream, or at the nicking site of said forward template, and does not nick within said target sequence;
c) providing a second nicking enzyme that is capable of nicking upstream, downstream, or at the nicking site of said reverse template and does not nick within said target sequence; and
d) providing a DNA polymerase;
under essentially isothermal conditions, wherein amplification is performed by multiple cycles of said polymerase extending said forward and reverse templates along said target sequence producing a double-stranded nicking site, and said nicking enzymes nicking at said nicking sites, or amplified copies of said sites, producing an amplification product.

43. A method for amplifying a single-stranded nucleic acid target sequence, comprising

a) contacting a target nucleic acid comprising a single-stranded target sequence with a reverse template, wherein said reverse template comprises a nucleic acid sequence comprising a recognition region at the 3' end that is complementary to the 3' end of the target sequence, a nicking enzyme binding site and a nicking site upstream of said recognition region, and a stabilizing region upstream of said nicking site, wherein the portion of the nucleic acid sequence that is complementary to the 3' end of the target sequence is 8-15 nucleotides in length;
b) providing a first nicking enzyme that is capable of nicking at the nicking site of said reverse template, and does not nick within said target sequence;
c) providing a DNA polymerase under conditions wherein said polymerase extends said reverse template along said target sequence;
d) contacting said extended reverse template with a forward template, wherein said forward template comprises a recognition region at the 3'end that is complementary to the 3' end of the extended reverse template, a nicking enzyme binding site and a nicking site upstream of said recognition region, and a stabilizing region upstream of said nicking site, wherein the portion of the nucleic acid sequence that is complementary to the 3' end of the target antisense strand is 8-15 nucleotides in length;
e) providing a second nicking enzyme that is capable of nicking at the nicking site of said forward template and does not nick within said target sequence or within the complement of said target sequence;
wherein the amplification is conducted under essentially isothermal conditions wherein amplification is performed by multiple cycles of said polymerase extending said forward and reverse templates along said target sequence, producing double-stranded nicking sites, and said nicking enzymes nicking at said nicking sites, producing an amplification product.

44. The method of paragraph 42 or 43, wherein said DNA polymerase is a thermophilic polymerase.
45. The method of paragraph 42 or 43, wherein said polymerase is selected from the group consisting of Bst (large fragment), 9°N, $Vent_R$® (exo-) DNA Polymerase, Therminator, and Therminator II.
46. The method of paragraph 42 or 43, wherein said polymerase is Bst(large fragment).
47. The method of paragraph 42 or 43, wherein said nicking enzymes nick downstream of the nicking enzyme

binding site.
48. The method of paragraph 42 or 43, wherein said forward and reverse templates comprise nicking enzyme binding sites recognized by the same nicking enzyme and said first and said second nicking enzymes are the same.
49. The method of paragraph 42 or 43, wherein said nicking enzymes are selected from the group consisting of Nt.BspQI, Nb.BbvCi, Nb.BsmI, Nb.BsrDI, Nb.BtsI, Nt.AlwI, Nt.BbvCI, Nt.BstNBI, Nt.CviPII, Nb.Bpu10I, and Nt.Bpu10I.
50. The method of paragraph 42 or 43, wherein said target sequence comprises from 1 to 5 nucleotides more than the sum of the nucleotides of said forward template recognition region and said reverse template recognition region.
51. The method of paragraph 42 or 43, wherein said target sequence comprises I nucleotide more than the sum of the nucleotides of said forward template recognition region and said reverse template recognition region.
52. The method of paragraph 42 or 43, wherein said target sequence comprises 2 nucleotides more than the sum of the nucleotides of said forward template recognition region and said reverse template recognition region.
53. The method of paragraph 42, wherein the target DNA molecule is selected from the group consisting of genomic DNA, plasmid, mitochondrial, and viral DNA.
54. The method of paragraph 43, wherein the target nucleic acid is selected from the group consisting of viral DNA, messenger RNA, microRNA, and microRNA precursors.
55. The method of paragraph 42 or 43, wherein the forward template is provided at the same concentration as the reverse template.
56. The method of paragraph 42 or 43, wherein one of the forward or reverse templates is provided at a ratio to the other template at the range of ratios of 1:100 to 100:1
57. The method of paragraph 43, further comprising a second polymerase.
58. The method of paragraph 57, wherein at least one of said polymerases comprises reverse transcriptase activity.
59. The method of paragraph 42 or 43, wherein the amplification is conducted between 54°C and 60°C.
60. The method of paragraph 42 or 43, wherein the amplification reaction is held at a constant temperature for 1 to 10 minutes.
61. The method of paragraph 42 or 43, further comprising detecting the amplification product.
62. The method of paragraph 42 or 43, wherein said amplification product is detected by a method selected from the group consisting of gel electrophoresis, mass spectrometry, SYBR I fluorescence, SYBR II fluorescence, SYBR Gold, Pico Green, TOTO-3, intercalating dye detection, FRET, molecular beacon detection, surface capture, capillary electrophoresis, incorporation of labeled nucleotides to allow detection by capture, fluorescence polarization, and lateral flow capture.
63. The method of paragraph 42 or 43, wherein at least two target sequences are capable of being amplified.
64. The method of paragraph 42 or 43, wherein said amplification products are detected on a solid surface.
65. The method of paragraph 42 or 43, wherein at least one capture probe is immobilized on a solid surface.
66. The method of paragraph 42 or 43, wherein at least one of said templates comprises a spacer, blocking group, or a modified nucleotide.
67. A method for amplifying a double-stranded nucleic acid target sequence, comprising

a) contacting a target DNA molecule comprising a double-stranded target sequence, having a sense strand and an antisense strand, with a forward template and a reverse template, wherein

i) said forward template comprises a nucleic acid sequence comprising a recognition region at the 3' end that is complementary to the 3' end of the target sequence antisense strand; a nicking enzyme binding site and a nicking site upstream of said recognition region and a stabilizing region upstream of said nicking site;
ii) said reverse template comprises a nucleotide sequence comprising a recognition region at the 3' end that is complementary to the 3' end of the target sequence sense strand, a nicking enzyme binding site and a nicking site upstream of said recognition region, and a stabilizing region upstream of said nicking site; and
iii) said target sequence comprises from 1 to 5 nucleotides more than the sum of the nucleotides of said forward template recognition region and said reverse template recognition region;

b) providing a first nicking enzyme that is capable of nicking at the nicking site of said forward template, and does not nick within said target sequence;
c) providing a second nicking enzyme that is capable of nicking at the nicking site of said reverse template and does not nick within said target sequence; and
d) providing a DNA polymerase;

under essentially isothermal conditions, wherein amplification is performed by multiple cycles of said polymerase

extending said forward and reverse templates along said target sequence producing a double-stranded nicking site, and said nicking enzymes nicking at said nicking sites, or amplified copies of said sites, producing an amplification product.

68. A method for amplifying a single-stranded nucleic acid target sequence, comprising

a) contacting a target nucleic acid comprising a single-stranded target sequence with a reverse template, wherein said reverse template comprises a nucleic acid sequence comprising a recognition region at the 3' end that is complementary to the 3' end of the target sequence, a nicking enzyme binding site and a nicking site upstream of said recognition region, and a stabilizing region upstream of said nicking site, wherein the portion of the nucleic acid sequence that is complementary to the 3' end of the target sequence is 8-15 nucleotides in length;
b) providing a first nicking enzyme that is capable of nicking at the nicking site of said reverse template, and does not nick within said target sequence;
c) providing a DNA polymerase under conditions wherein said polymerase extends said reverse template along said target sequence;
d) contacting said extended reverse template with a forward template, wherein said forward template comprises a recognition region at the 3'end that is complementary to the 3' end of the extended reverse template, a nicking enzyme binding site and a nicking site upstream of said recognition region, and a stabilizing region upstream of said nicking site, wherein said target sequence comprises from 1 to 5 nucleotides more than the sum of the nucleotides of said forward template recognition region and said reverse template recognition region;
e) providing a second nicking enzyme that is capable of nicking at the nicking site of said forward template and does not nick within said target sequence or within the complement of said target sequence;

wherein the amplification is conducted under essentially isothermal conditions wherein amplification is performed by multiple cycles of said polymerase extending said forward and reverse templates along said target sequence, producing double-stranded nicking sites, and said nicking enzymes nicking at said nicking sites, producing an amplification product.

69. The method of paragraph 67 or 68, wherein said target sequence comprises l nucleotide more than the sum of the nucleotides of said forward template recognition region and said reverse template recognition region.

70. The method of paragraph 67 or 68, wherein said target sequence comprises 2 nucleotides more than the sum of the nucleotides of said forward template recognition region and said reverse template recognition region.

71. The method of paragraph 67 or 68, wherein said target sequence comprises 3 nucleotides more than the sum of the nucleotides of said forward template recognition region and said reverse template recognition region.

72. A method for amplifying a double-stranded nucleic acid target sequence, comprising

a) contacting a target DNA molecule comprising a double-stranded target sequence, having a sense strand and an antisense strand, with a forward template and a reverse template, wherein
b) said forward template comprises a nucleic acid sequence comprising a recognition region at the 3' end that is complementary to the 3' end of the target sequence antisense strand; a nicking enzyme binding site and a nicking site upstream of said recognition region and a stabilizing region upstream of said nicking site;
c) said reverse template comprises a nucleotide sequence comprising a recognition region at the 3' end that is complementary to the 3' end of the target sequence sense strand, a nicking enzyme binding site and a nicking site upstream of said recognition region, and a stabilizing region upstream of said nicking site;
d) providing a first nicking enzyme that is capable of nicking at the nicking site of said forward template, and does not nick within said target sequence;
e) providing a second nicking enzyme that is capable of nicking at the nicking site of said reverse template and does not nick within said target sequence; and
f) providing a DNA polymerase;
under essentially isothermal conditions, wherein amplification is performed by multiple cycles of said polymerase extending said forward and reverse templates along said target sequence producing a double-stranded nicking site, and said nicking enzymes nicking at said nicking sites, or amplified copies of said sites, producing an amplification product.

73. A method for amplifying a double-stranded nucleic acid target sequence, comprising

a) contacting a target DNA molecule comprising a double-stranded target sequence, having a sense strand and an antisense strand, with a forward template and a reverse template, wherein
b) said forward template comprises a nucleic acid sequence comprising a recognition region at the 3' end that is complementary to the 3' end of the target sequence antisense strand; a nicking enzyme binding site and a

nicking site upstream of said recognition region and a stabilizing region upstream of said nicking site;

ii) said reverse template comprises a nucleotide sequence comprising recognition region at the 3' end that is complementary to the 3' end of the target sequence sense strand, a nicking enzyme binding site and a nicking site upstream of said recognition region, and a stabilizing region upstream of said nicking site;

c) providing a first nicking enzyme that is capable of nicking upstream, downstream, or at the nicking site of said forward template, and does not nick within said target sequence;
d) providing a second nicking enzyme that is capable of nicking upstream, downstream, or at the nicking site of said reverse template and does not nick within said target sequence; and
e) providing a DNA polymerase;

under essentially isothermal conditions, wherein amplification is performed by multiple cycles of said polymerase extending said forward and reverse templates along said target sequence producing a double-stranded nicking site, and said nicking enzymes nicking at said nicking sites, or amplified copies of said sites, producing an amplification product.
74. A method for amplifying a double-stranded nucleic acid target sequence, comprising

a) contacting a target DNA molecule comprising a double-stranded target sequence, having a sense strand and an antisense strand, with a forward template and a reverse template, wherein
b) said forward template comprises a nucleic acid sequence comprising a recognition region at the 3' end that is complementary to the 3' end of the target sequence antisense strand; a nicking enzyme binding site and a nicking site upstream of said recognition region and a stabilizing region upstream of said nicking site;
c) said reverse template comprises a nucleotide sequence comprising a recognition region at the 3' end that is complementary to the 3' end of the target sequence sense strand, a nicking enzyme binding site and a nicking site upstream of said recognition region, and a stabilizing region upstream of said nicking site;
d) providing a first nicking enzyme that is capable of nicking at the nicking site of said forward template, and does not nick within said target sequence;
e) providing a second nicking enzyme that is capable of nicking at the nicking site of said reverse template and does not nick within said target sequence; and
f) providing a DNA polymerase;

under essentially isothermal conditions, wherein amplification is performed by multiple cycles of said polymerase extending said forward and reverse templates along said target sequence producing a double-stranded nicking site, and said nicking enzymes nicking at said nicking sites, or amplified copies of said sites, producing an amplification product, wherein at least a 1E + 7 fold amplification of a 22-35 nucleotide long target sequence is obtained when the amplification reaction is run for twelve minutes.
75. A method for amplifying a double-stranded nucleic acid target sequence, comprising

a) contacting a target DNA molecule comprising a double-stranded target sequence, having a sense strand and an antisense strand, with a forward template and a reverse template, wherein
b) said forward template comprises a nucleic acid sequence comprising a recognition region at the 3' end that is complementary to the 3' end of the target sequence antisense strand; a nicking enzyme binding site and a nicking site upstream of said recognition region and a stabilizing region upstream of said nicking site, wherein the portion of the nucleic acid sequence that is complementary to the 3' end of the target antisense strand is 8-15 nucleotides in length;
c) said reverse template comprises a nucleotide sequence comprising recognition region at the 3' end that is complementary to the 3' end of the target sequence sense strand, a nicking enzyme binding site and a nicking site upstream of said recognition region, and a stabilizing region upstream of said nicking site, wherein the portion of the nucleic acid sequence that is complementary to the 3' end of the target antisense strand is 8-15 nucleotides in length;
d) providing a first nicking enzyme that is capable of nicking upstream, downstream, or at the nicking site of said forward template, and does not nick within said target sequence;
e) providing a second nicking enzyme that is capable of nicking upstream, downstream, or at the nicking site of said reverse template and does not nick within said target sequence; and
f) providing a DNA polymerase;

under essentially isothermal conditions, wherein amplification is performed by multiple cycles of said polymerase

extending said forward and reverse templates along said target sequence producing a double-stranded nicking site, and said nicking enzymes nicking at said nicking sites, or amplified copies of said sites, producing an amplification product, wherein at least a 1E + 7 fold amplification of a 22-35 nucleotide long target sequence is obtained when the amplification reaction is run for twelve minutes.

76. A kit for amplifying a nucleic acid target sequence, comprising

a) a DNA polymerase;
b) a first template for nucleic acid amplification, comprising a recognition region at the 3' end that is complementary to the 3' end of a target sequence sense strand; a nicking enzyme binding site and a nicking site upstream of said recognition region; and a stabilizing region upstream of said nicking site, wherein the portion of the nucleic acid sequence that is complementary to the 3' end of the target sequence sense strand is 8-15 nucleotides in length;
c) a second template for nucleic acid amplification, comprising a recognition region at the 3' end that is complementary to the 3' end of the complement of the target sequence sense strand; a nicking enzyme binding site and a nicking site upstream of said recognition region; and a stabilizing region upstream of said nicking site, wherein the portion of the nucleic acid sequence that is complementary to the 3' end of the complement of the target sequence sense strand is 8-15 nucleotides in length; and
d) one or two thermostable nicking enzymes, wherein either one enzyme is capable of nicking at the nicking site of said first and said second templates, or a first enzyme is capable of nicking at the nicking site of said first primer and a second enzyme is capable of nicking at the enzyme site of said second primer.

77. The kit of paragraph 76, wherein said target sequence comprises from 1 to 5 nucleotides more than the sum of the nucleotides of said first template recognition region and said second template recognition region.
78. The kit of paragraph 76, wherein said polymerase, nicking enzymes, and templates are in a container.
79. The kit of paragraph 76, wherein said polymerase, nicking enzymes, and templates are in two containers.
80. The kit of paragraph 76, wherein said polymerase and nicking enzymes are in a first container, and said templates are in a second container.
81. The kit of paragraph 76, wherein said polymerase, nicking enzymes, and templates are lyophilized.
82. The kit of paragraph 76, further comprising instructions for following the method of amplification.
83. The kit of paragraph 76, further comprising a cuvette.
84. The kit of paragraph 76 further comprising a lateral flow device or dipstick.
85. The kit of paragraph 84, wherein said lateral flow device or dipstick further comprises a capture probe.
86. The kit of paragraph 76, further comprising a detector component selected from the group consisting of a fluorescent dye, colloidal gold particles, latex particles, a molecular beacon, and polystyrene beads.
87. The kit of paragraph 76, wherein at least one of said templates comprises a spacer, blocking group, or a modified nucleotide.
88. A kit for amplifying a nucleic acid target sequence, comprising

a) a DNA polymerase;
b) a first template for nucleic acid amplification, comprising a recognition region at the 3' end that is complementary to the 3' end of a target sequence sense strand; a nicking enzyme binding site and a nicking site upstream of said recognition region; and a stabilizing region upstream of said nicking site;
c) a second template for nucleic acid amplification, comprising a recognition region at the 3' end that is complementary to the 3' end of the complement of the target sequence sense strand; a nicking enzyme binding site and a nicking site upstream of said recognition region; and a stabilizing region upstream of said nicking site, wherein said target sequence comprises from 1 to 5 nucleotides more than the sum of the nucleotides of said first template recognition region and said second template recognition region; and
d) one or two thermostable nicking enzymes, wherein either one enzyme is capable of nicking at the nicking site of said first and said second templates, or a first enzyme is capable of nicking at the nicking site of said first primer and a second enzyme is capable of nicking at the enzyme site of said second primer.

89. The kit of paragraph 88, wherein the portion of the nucleic acid sequence of the first template that is complementary to the 3' end of the target sequence sense strand is 8-15 nucleotides in length, and the portion of the nucleic acid sequence of the second template that is complementary to the 3' end of the complement of the target sequence sense strand is 8-15 nucleotides in length.
90. The kit of paragraph 88, wherein said polymerase, nicking enzymes, and templates are in a container.
91. The kit of paragraph 88, wherein said polymerase, nicking enzymes, and templates are in two containers.
92. The kit of paragraph 88, wherein said polymerase and nicking enzymes are in a first container, and said templates

are in a second container.
93. The kit of paragraph 88, wherein said polymerase, nicking enzymes, and templates are lyophilized.
94. The kit of paragraph 88, further comprising instructions for following the method of amplification.
95. The kit of paragraph 88, further comprising a cuvette.
96. The kit of paragraph 88 further comprising a lateral flow device or dipstick.
97. The kit of paragraph 96, wherein said lateral flow device or dipstick further comprise a capture probe.
98. The kit of paragraph 88, further comprising a detector component selected from the group consisting of a fluorescent dye, colloidal gold particles, latex particles, a molecular beacon, and polystyrene beads.
99. The kit of paragraph 88, wherein at least one of said templates comprises a spacer, blocking group, or a modified nucleotide.

Claims

1. A method for amplifying a nucleotide sequence from a sample, comprising providing a sample containing a target nucleic acid having a target nucleotide sequence and combining said target nucleic acid with:

   (i) a polymerase,
   (ii) a first template that comprises a nucleic acid sequence comprising a first template recognition region at the 3' end that is complementary or substantially complementary to the 3' end of the complement of the first strand of the target nucleotide sequence, and a nicking enzyme binding site and nicking site upstream of the recognition region;
   (iii) a second template that comprises a nucleic acid sequence comprising a second template recognition region at the 3' end that is complementary or substantially complementary to the 3' end of the first strand of the target nucleotide sequence, and a nicking enzyme binding site and nicking site upstream of the recognition region; and
   (iv) one or more nicking enzymes capable of nicking at the nicking site of said first and second templates, wherein either one nicking enzyme is capable of nicking both of said templates, or each template is capable of being nicked by at least one of the nicking enzymes, wherein said one or more nicking enzymes do not nick within said target nucleotide sequence;
   and performing nicking and template extension reactions under isothermal conditions.

2. The method of claim 1, wherein the first template and second template each further comprise a stabilizing region 5' of said nicking site, optionally wherein said stabilizing region has a GC content of at least 40% and/or a length of at least 6 nucleotides.

3. The method of claims 1 or 2, wherein either:

   (i) the recognition region of the first and second templates is 8-12, 8-13 or 8-15 nucleotides in length; and/or
   (ii) there is an internal hairpin within said template sequence.

4. The method of claims 1-3, wherein the amplification reaction is run at about 37°C-85°C, 37°C -60°C, 54°C-60°C, 55°C-60°C, 58°C-60°C or 56°C-58°C, and wherein:

   (i) the template extension reactions comprise multiple cycles of the polymerase extending the first and second templates along the target nucleotide sequence;
   (ii) the template extension reactions produce a double-stranded nicking site; and/or
   (iii) the nicking reactions comprise the one or more nicking enzymes nicking at nicking sites.

5. The method of claims 1-4, wherein the sample is isolated from an animal, optionally wherein the sample comprises blood, bone marrow, mucus, lymph, hard tissues (e.g. liver, spleen, kidney, lung or ovary), biopsies, sputum, saliva, tears, faeces or urine.

6. The method of claims 1-4, wherein the target nucleotide sequence is:

   (i) amplified from a sample containing a spore, virus, cell, prokaryote or eukaryote, optionally wherein said target nucleotide sequence is detected on the outside of a spore without the need for lysis; or
   (ii) from an animal, plant, insect or bacterial genome.

7. The method of claims 1-4, wherein the target nucleic acid is present in:

(i) waste water, drinking water, air, milk, or other food; or
(ii) air, plant, soil or other materials suspected of containing biological organisms.

8. The method of any of claims 1-7, wherein the target nucleic acid is double stranded or single stranded, optionally wherein the target nucleic acid is:

(i) double-stranded DNA, e.g. wherein the target nucleic acid is selected from the group consisting of genomic DNA, plasmid DNA, viral DNA, mitochondrial DNA, and synthetic double-stranded DNA;
(ii) single-stranded DNA, e.g. wherein the target nucleic acid is selected from the group consisting of viral DNA, cDNA, and synthetic single-stranded DNA;
(iii) RNA, e.g. wherein the target nucleic acid is selected from the group consisting of messenger RNA, viral RNA, ribosomal RNA, transfer RNA, micro RNA, micro RNA precursor, and synthetic RNA.

9. The method of claim 8, wherein:

(i) the target nucleic acid is double stranded and wherein there is no initial heat denaturation step; and/or
(ii) the temperature of the amplification reaction is higher than the initial melting temperature of the template/target sequence complex.

10. The method of any preceding claim, wherein said one or more nicking enzymes are selected from the group of nicking enzymes shown in Table 3 or from the group of nicking enzymes consisting of Nt.BspQI, Nb.BbvCi, Nb.BsmI, Nb.BsrDI, Nb.BtsI, NtAlwI, NtBbvCI, NtBstNBI, Nt.CviPII, Nb.Bpu10I, and Nt.Bpu10I, preferably wherein the nicking enzyme is selected from the group consisting of Nt.Bst.NBI, Nb.BsmI and Nb.BsrDI.

11. The method of any of any preceding claim, further comprising detecting the amplification product, optionally wherein said amplification product is detected by a detection method selected from the group consisting of gel electrophoresis, mass spectrometry, SYBR I fluorescence, SYBR II fluorescence, SYBR Gold, Pico Green, TOTO-3, intercalating dye detection, fluorescence resonance energy transfer (FRET), molecular beacon detection, surface capture, capillary electrophoresis, incorporation of labeled nucleotides to allow detection by capture, fluorescence polarization, and lateral flow capture.

12. A kit for amplifying a target nucleotide sequence of a target nucleic acid, comprising

a) a DNA polymerase;
b) a first template that comprises a nucleic acid sequence comprising a first template recognition region at the 3' end that is complementary or substantially complementary to the 3' end of the complement of the first strand of the target nucleotide sequence, and a nicking enzyme binding site and nicking site upstream of the recognition region;
c) a second template that comprises a nucleic acid sequence comprising a second template recognition region at the 3' end that is complementary or substantially complementary to the 3' end of the first strand of the target nucleotide sequence, and a nicking enzyme binding site and nicking site upstream of the recognition region; and
d) one or more nicking enzymes capable of nicking at the nicking site of said first and second templates, wherein either one nicking enzyme is capable of nicking both of said templates, or each template is capable of being nicked by at least one of the nicking enzymes, wherein said one or more nicking enzymes do not nick within said target nucleotide sequence.

13. The kit of claim 12, wherein said target nucleic acid, first and second templates, and one or more nicking enzymes are as defined in any preceding claim.

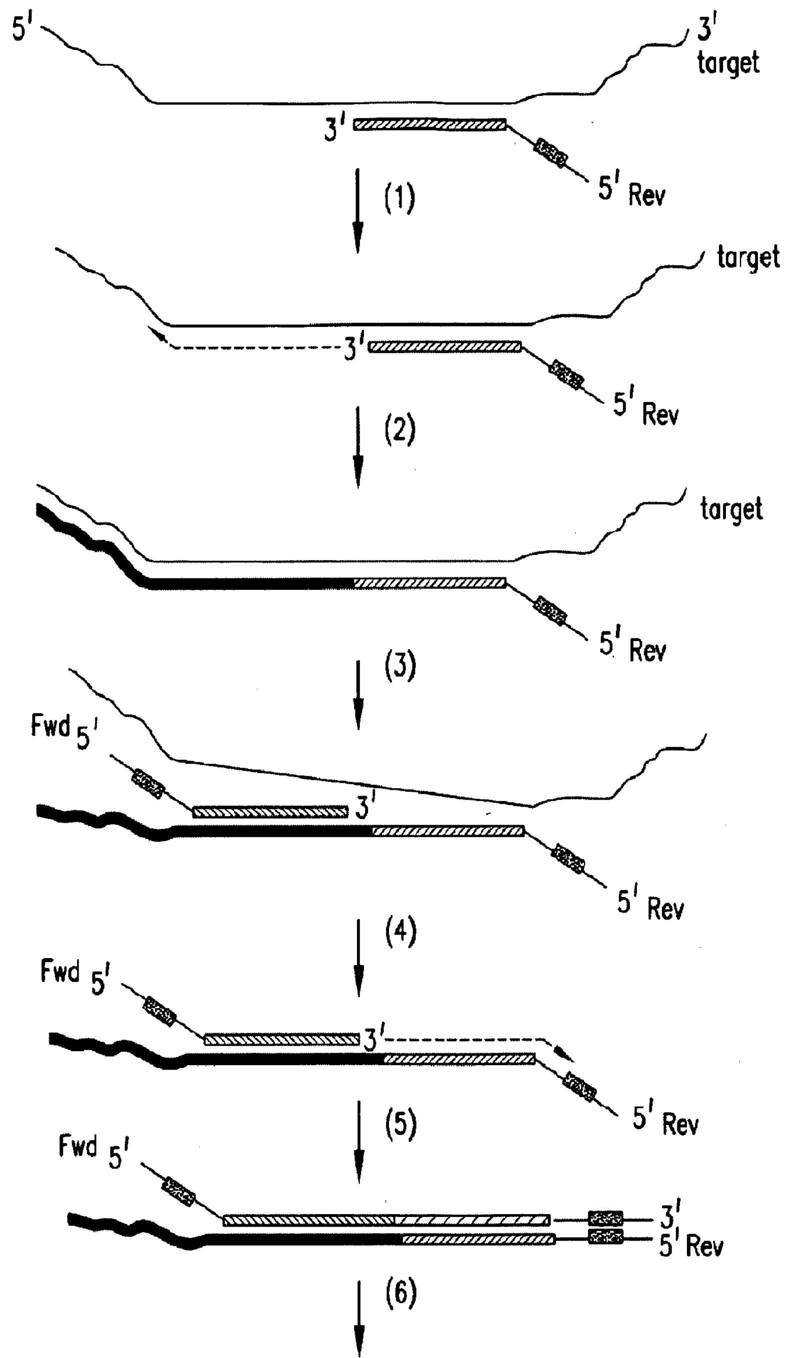


FIG.1A

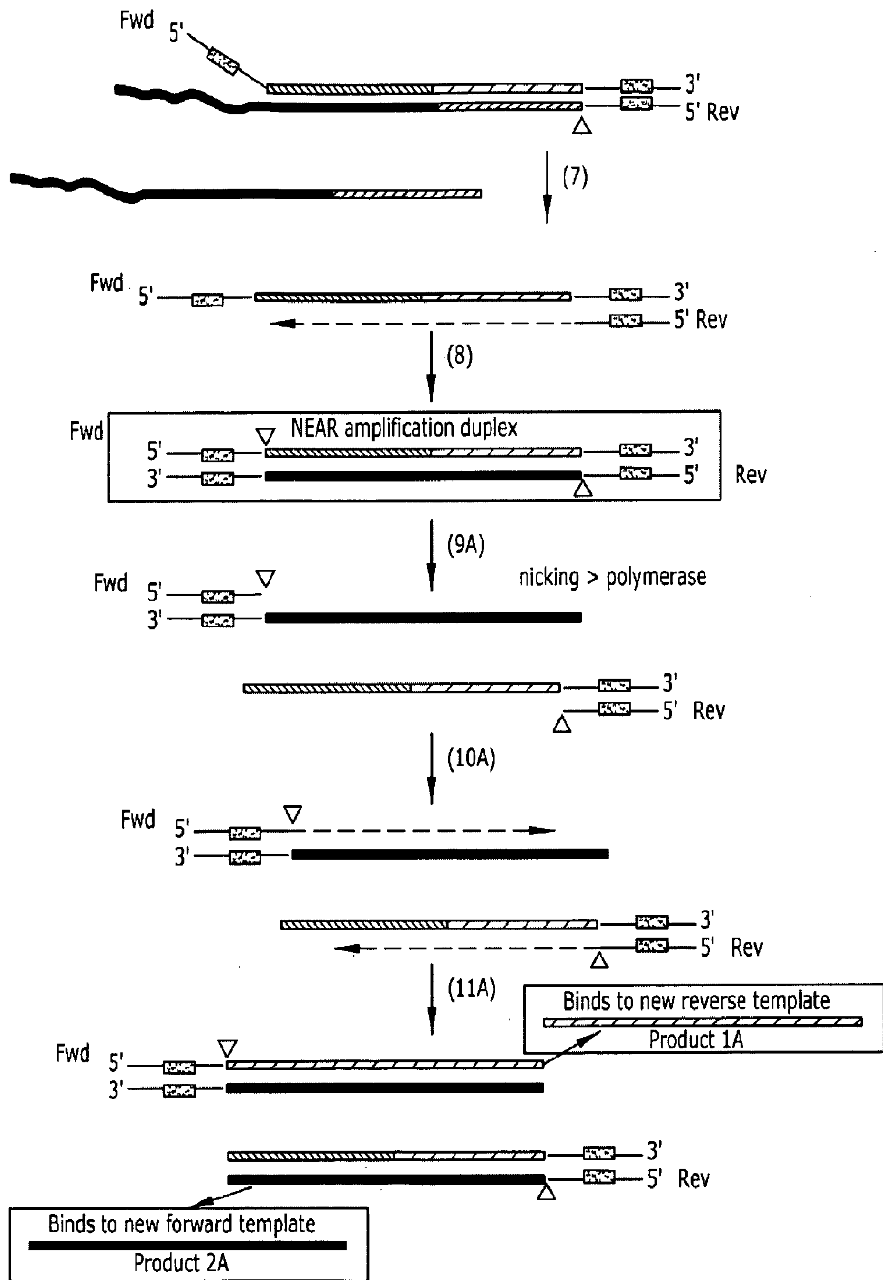


FIG. 1B

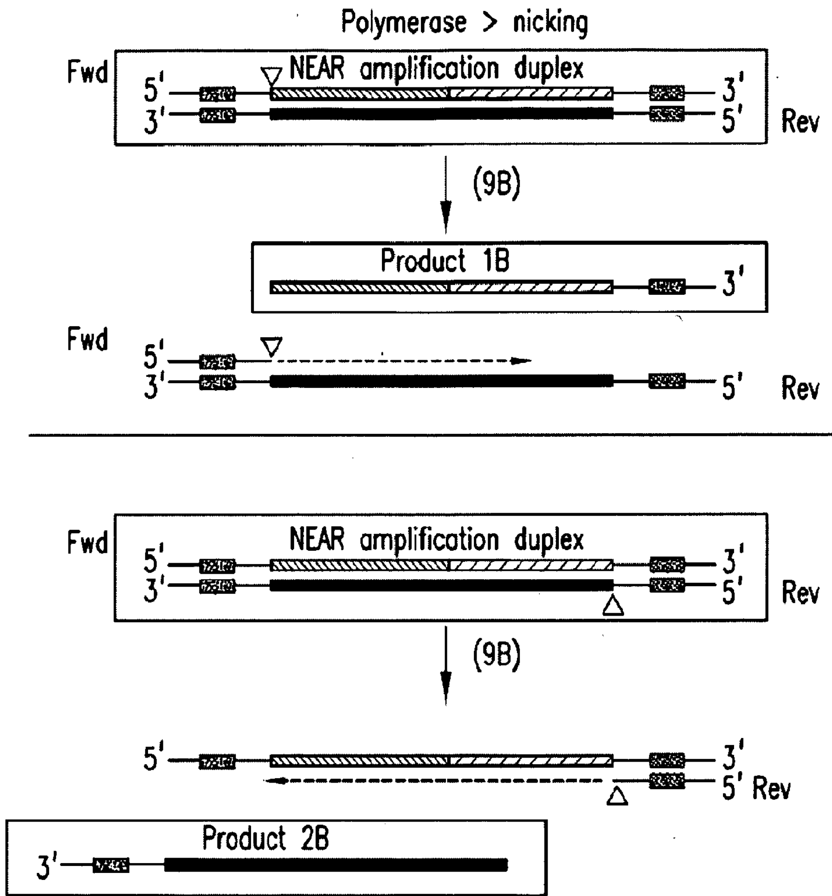


FIG.1C

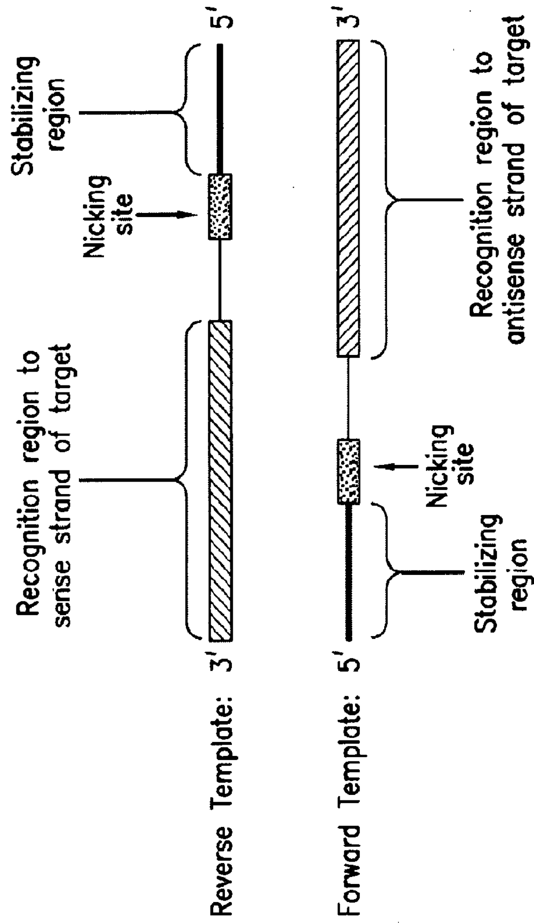


FIG.1D

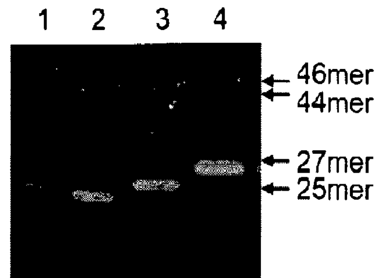


FIG.2

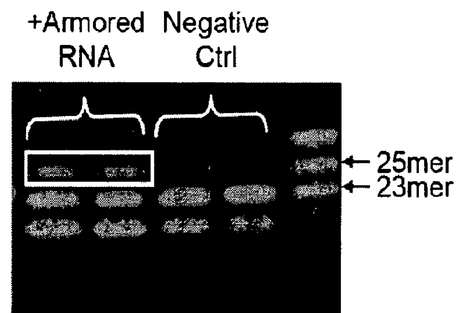
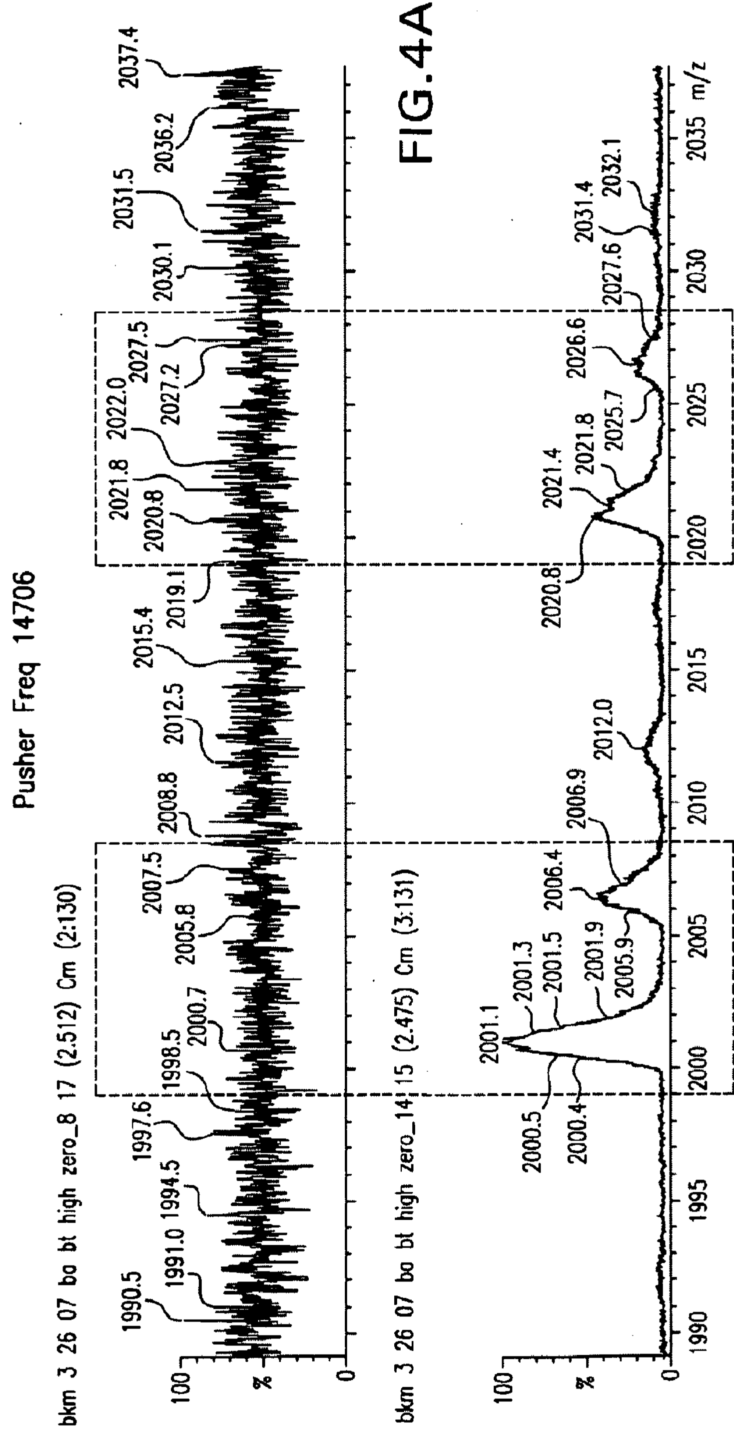


FIG.3


Pusher Freq 14706
bkm 3 26 07 bo bt high zero_8 17 (2.512) Cm (2:130)
bkm 3 26 07 bo bt high zero_14 15 (2.475) Cm (3:131)
m/z
FIG.4A
FIG.4B

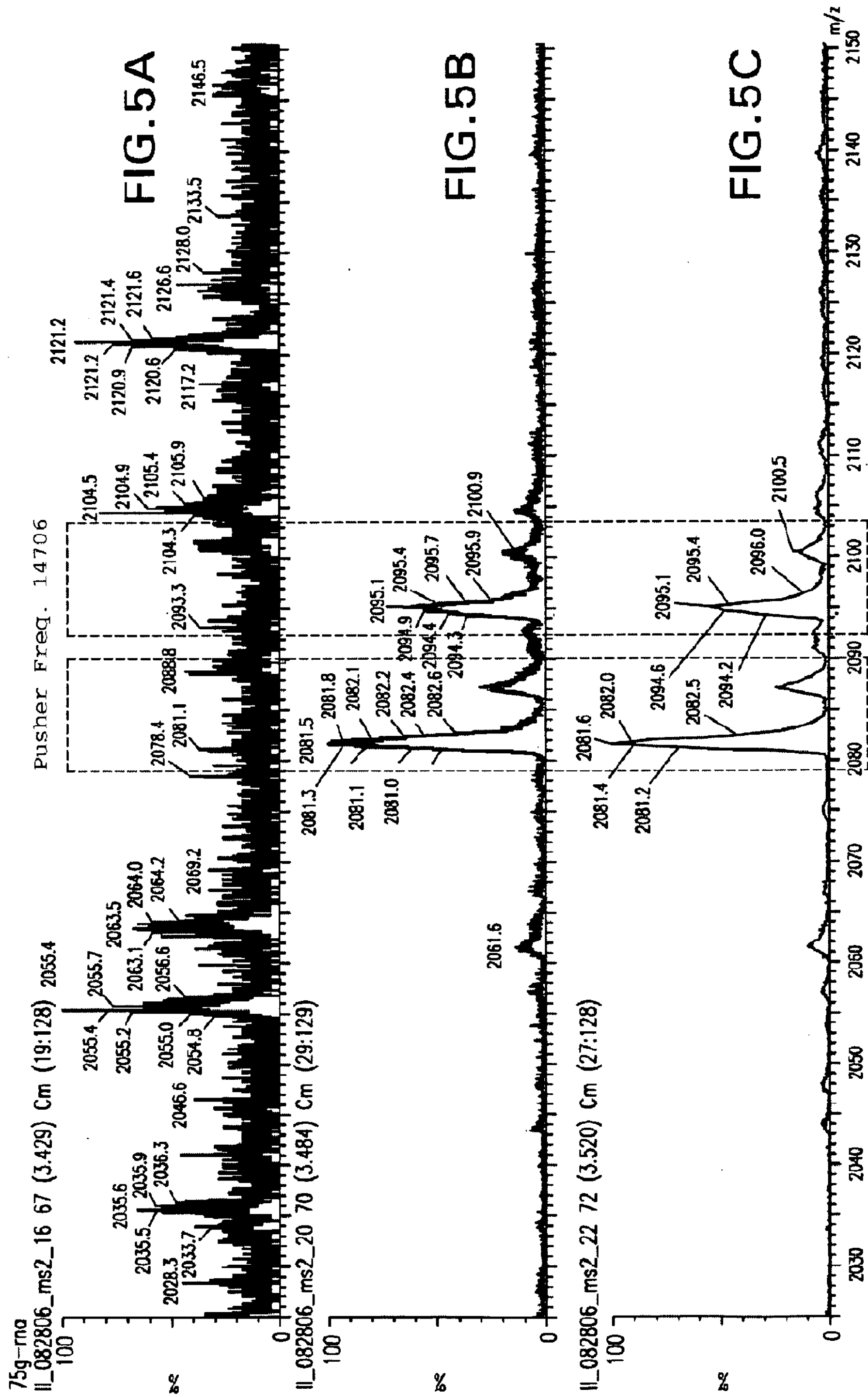


FIG.5A
FIG.5B
FIG.5C
Pusher Freq. 14706
75g-rna
II_082806_ms2_16 67 (3.429) Cm (19:128)
II_082806_ms2_20 70 (3.484) Cm (29:129)
II_082806_ms2_22 72 (3.520) Cm (27:128)
100
%
0
m/z
2030
2040
2050
2060
2070
2080
2090
2100
2110
2120
2130
2140
2150

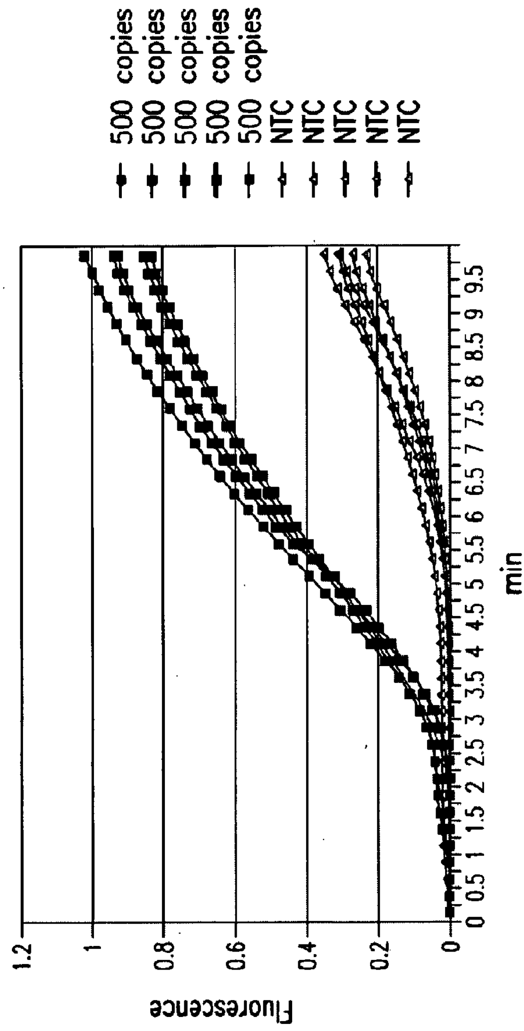


FIG.6

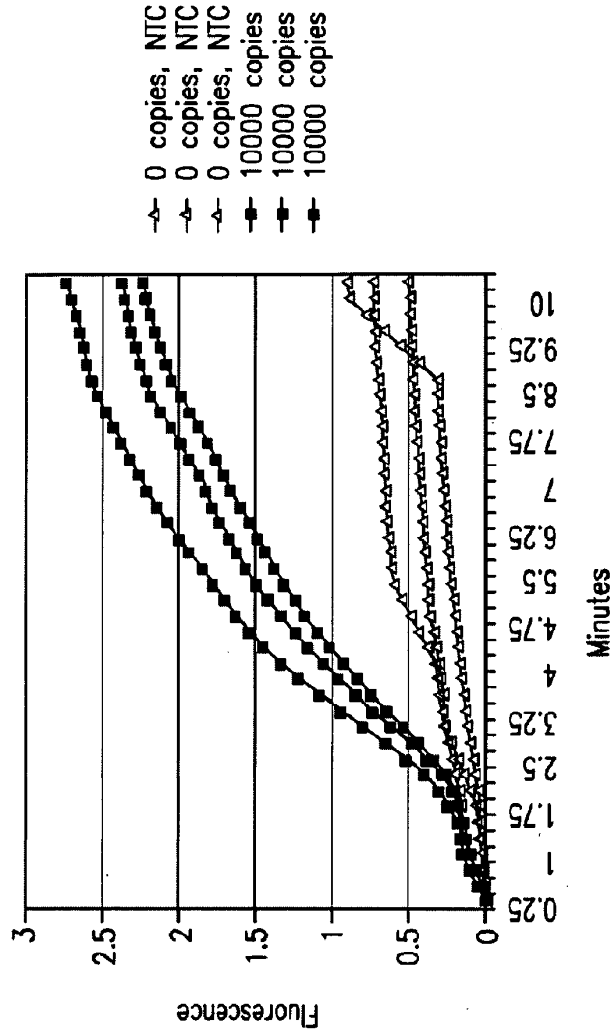


FIG.7

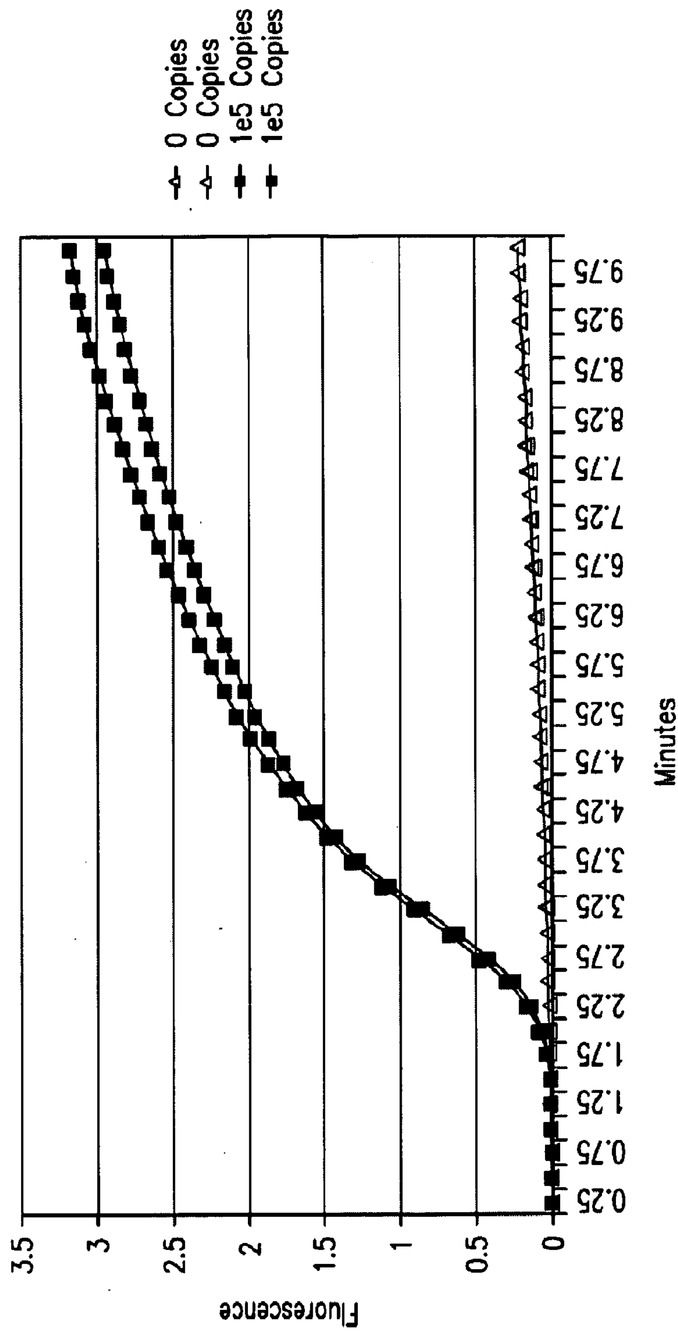


FIG.8

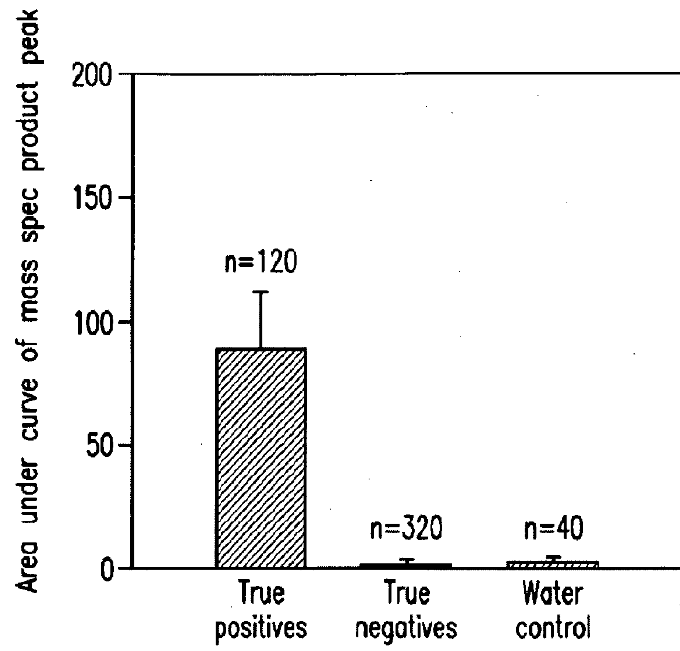


FIG.9

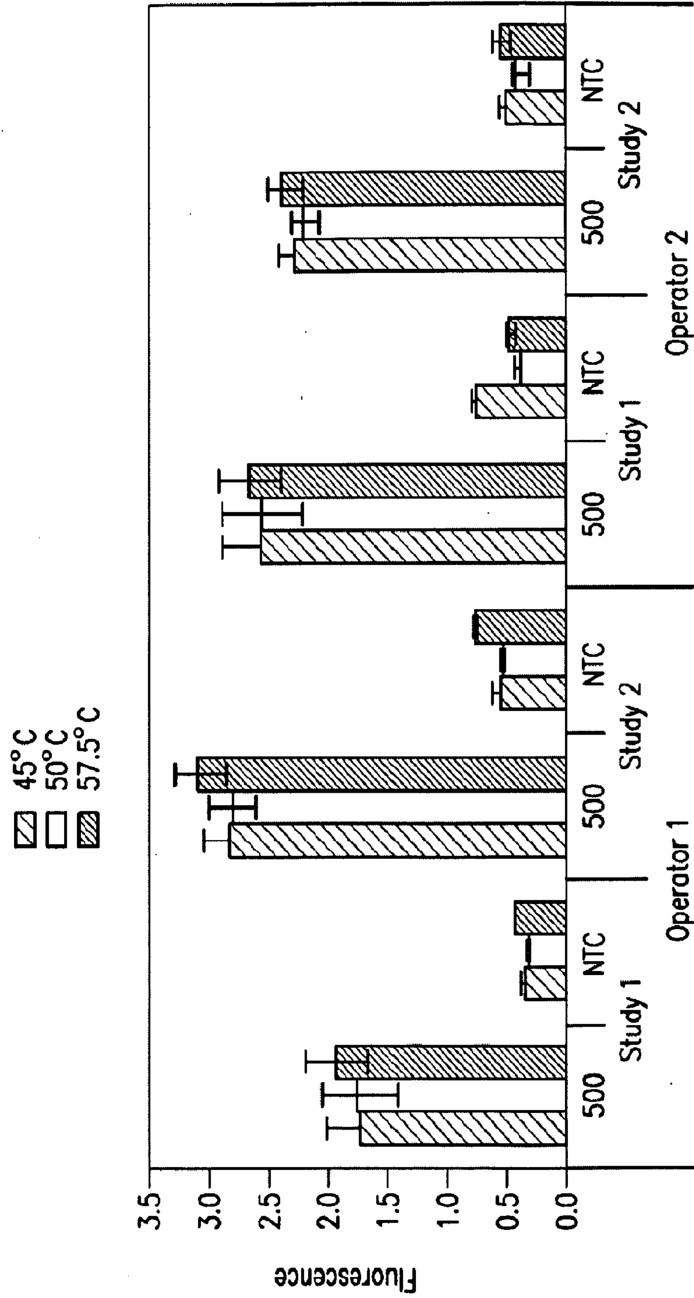


FIG.10

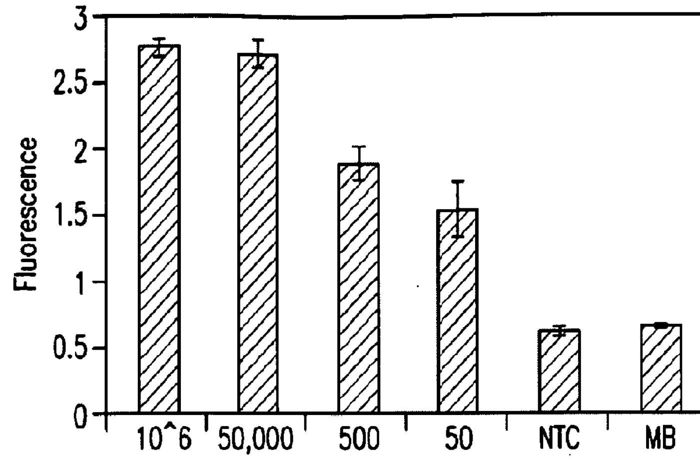


FIG.11

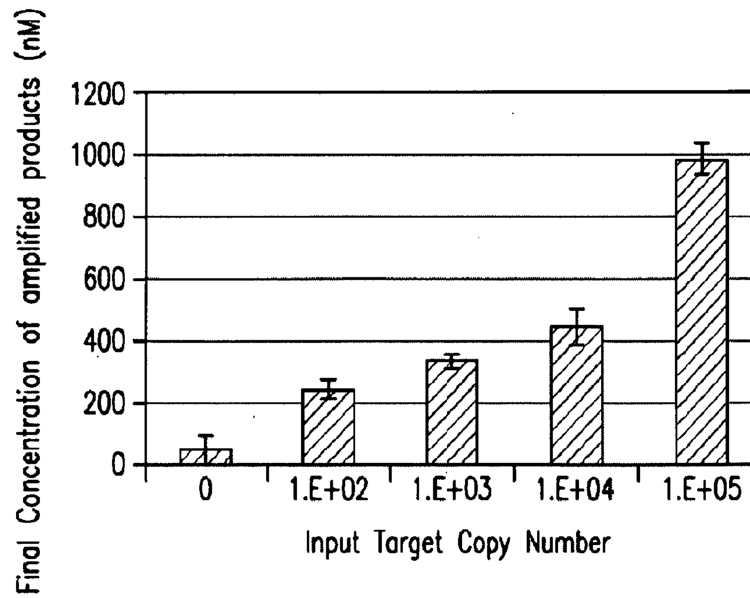


FIG.12

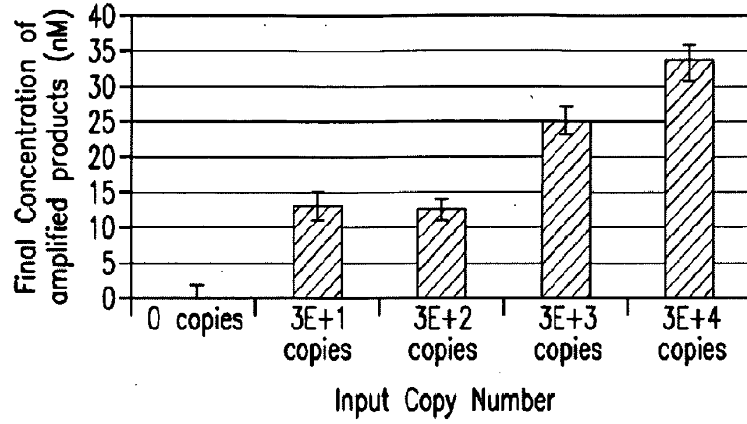


FIG.13

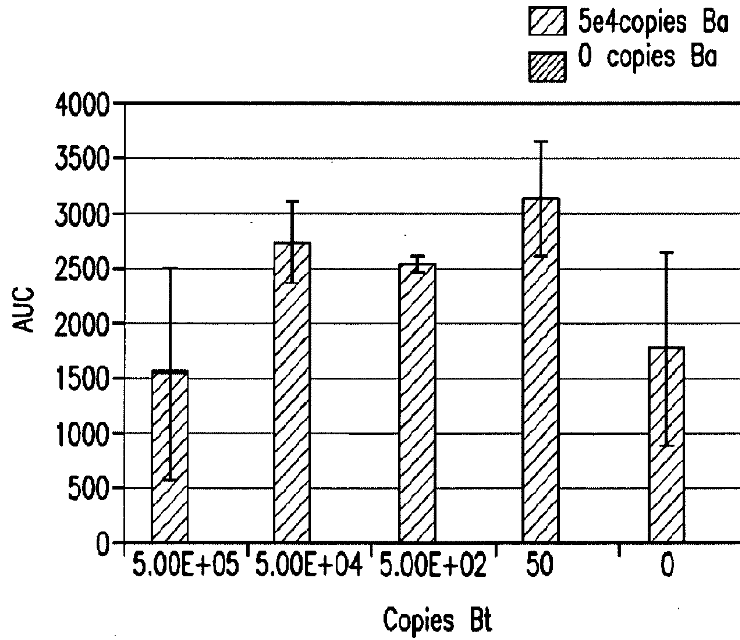


FIG.14

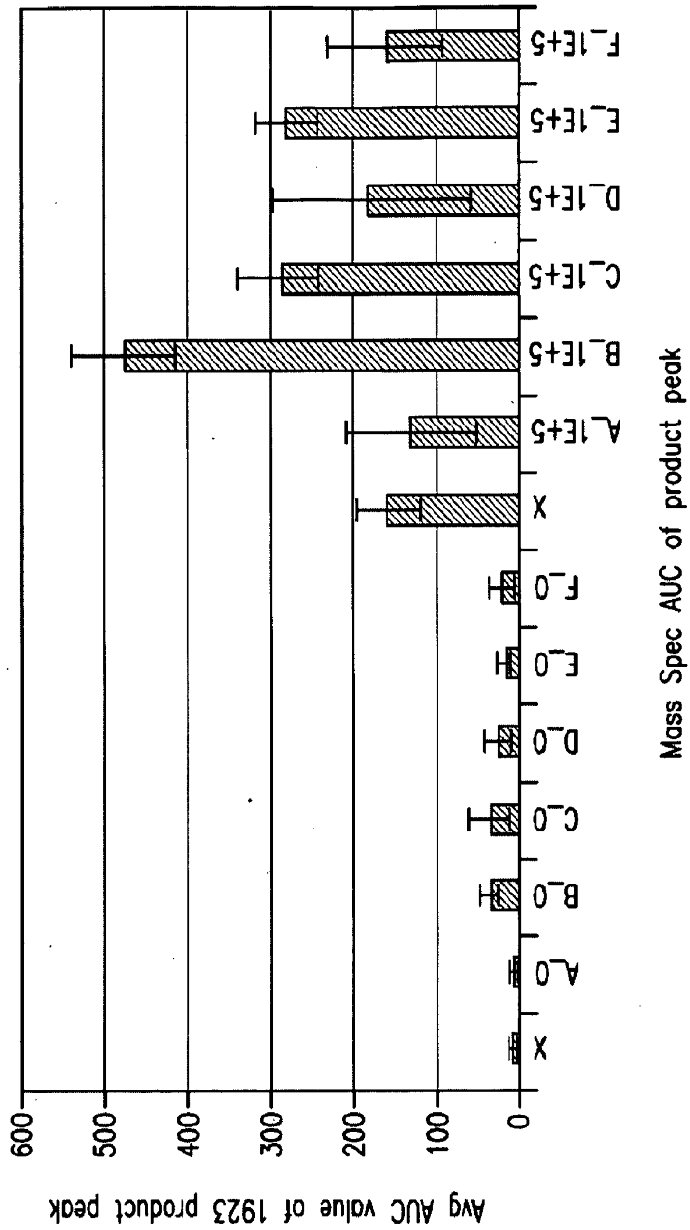


FIG.15

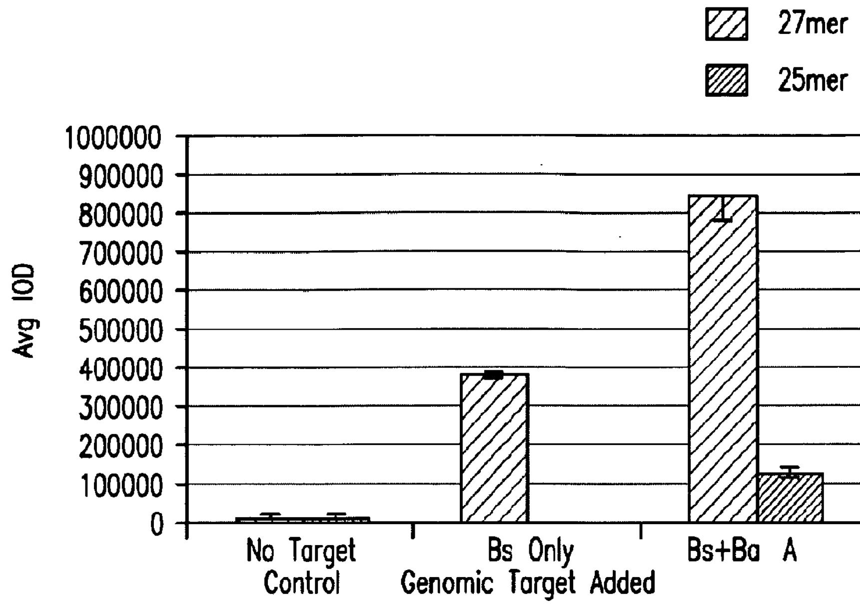


FIG.16

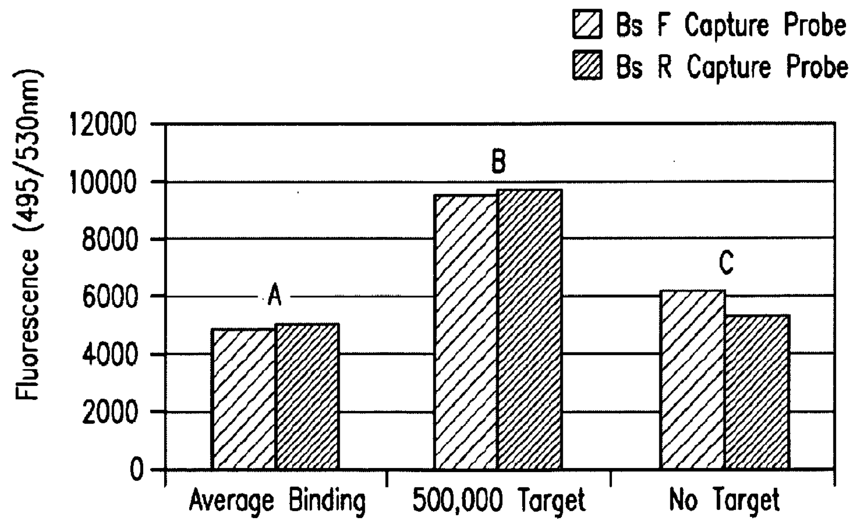


FIG.20

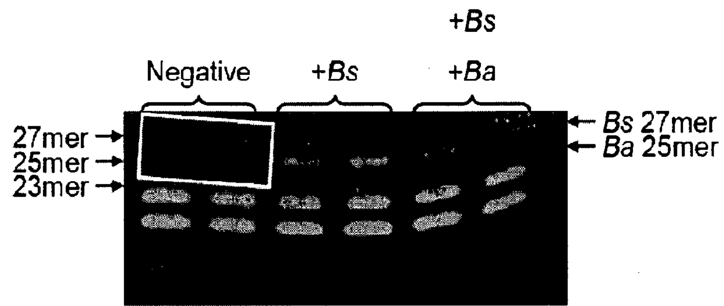


FIG.17

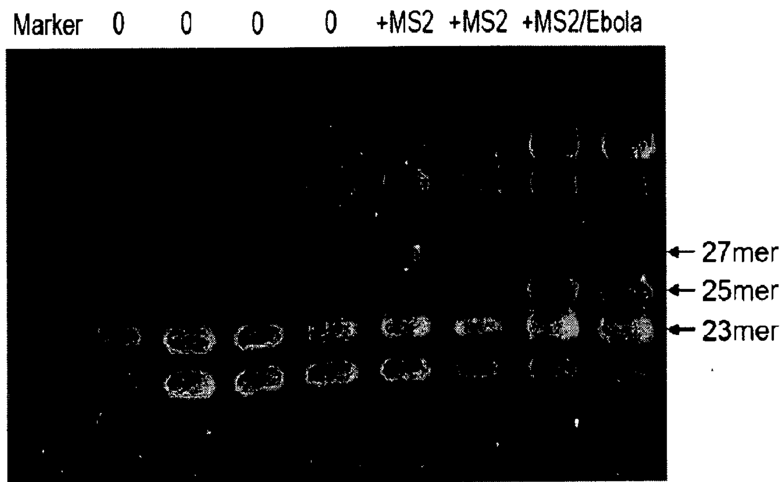


FIG.18

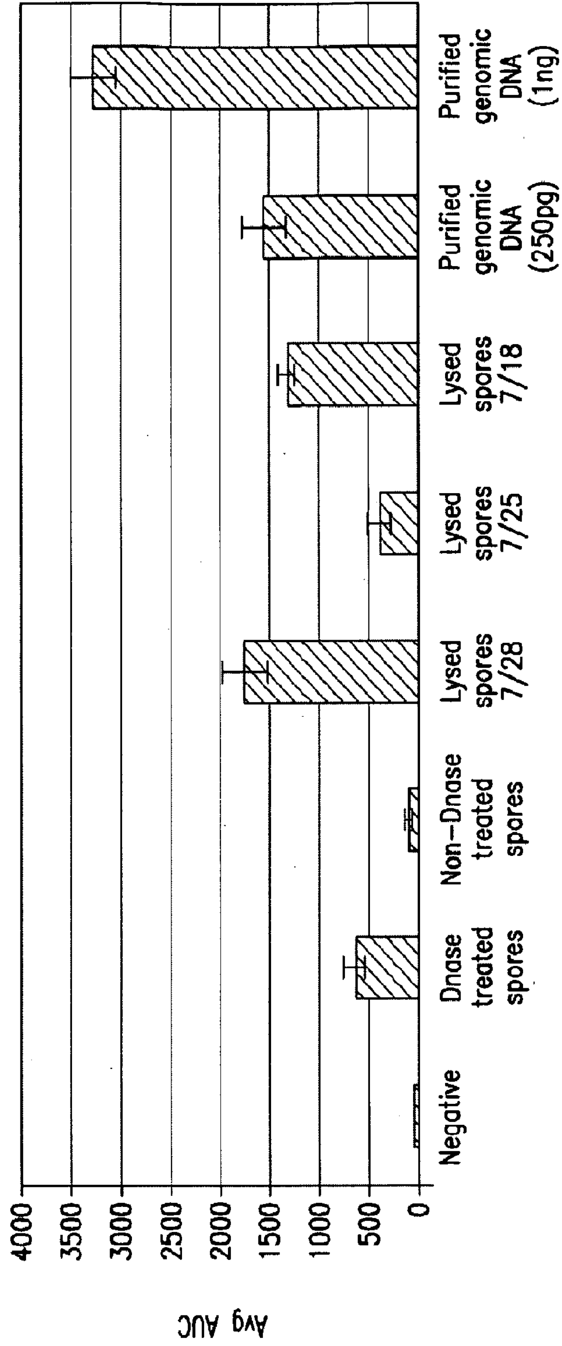


FIG.19

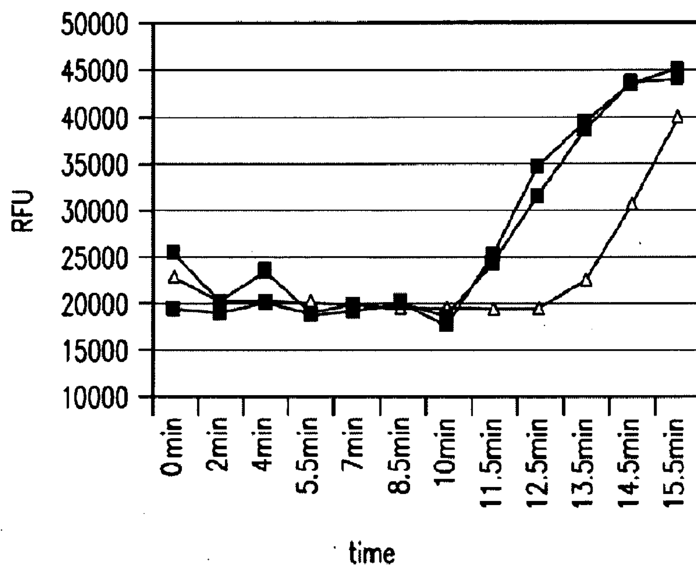


FIG.21

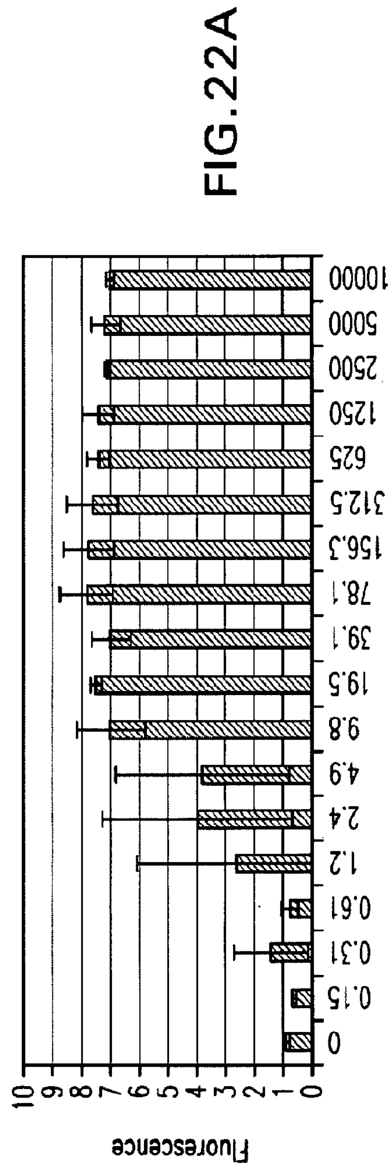


FIG.22A

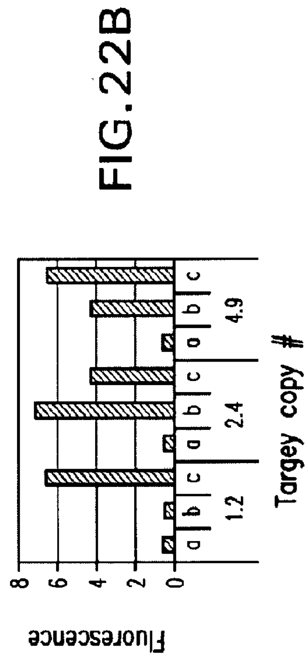


FIG.22B

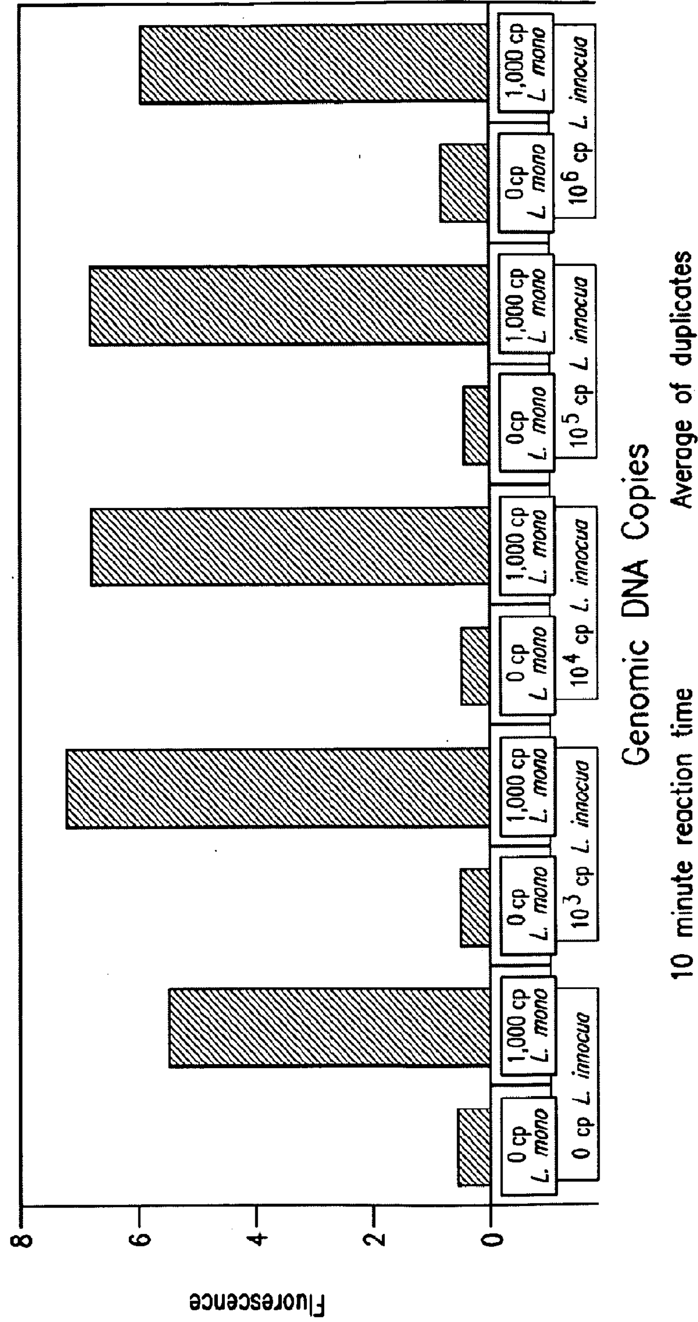


FIG.23

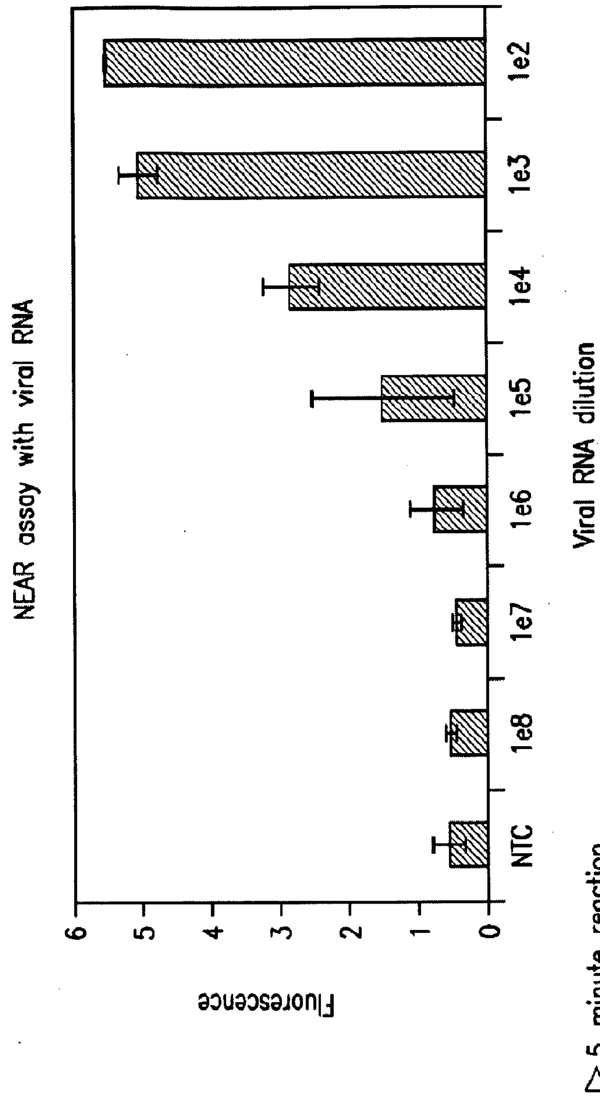


FIG.24

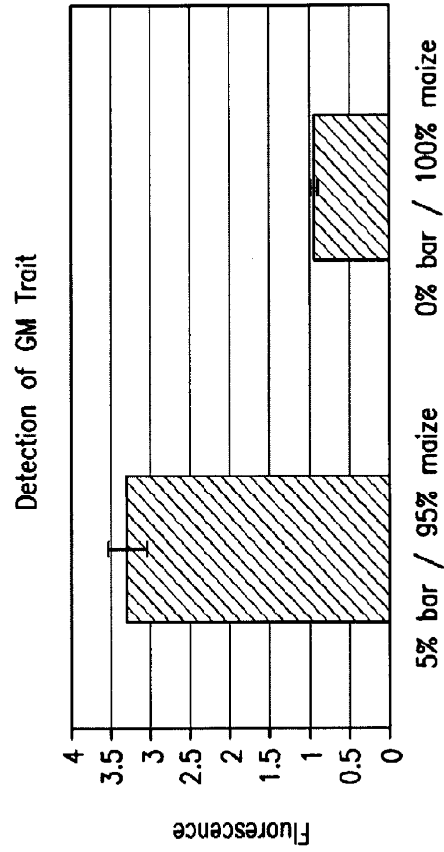


FIG.25

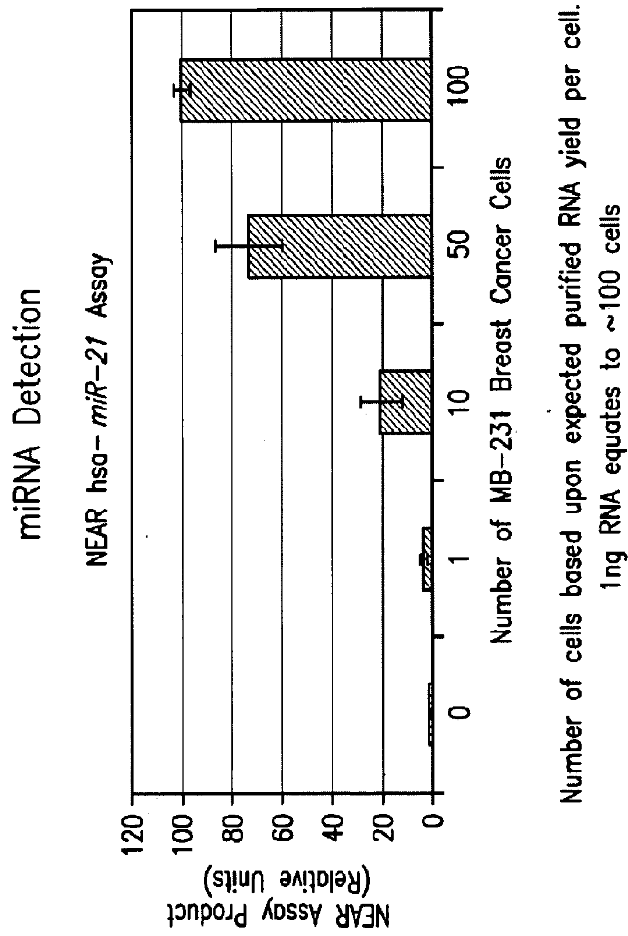


FIG.26

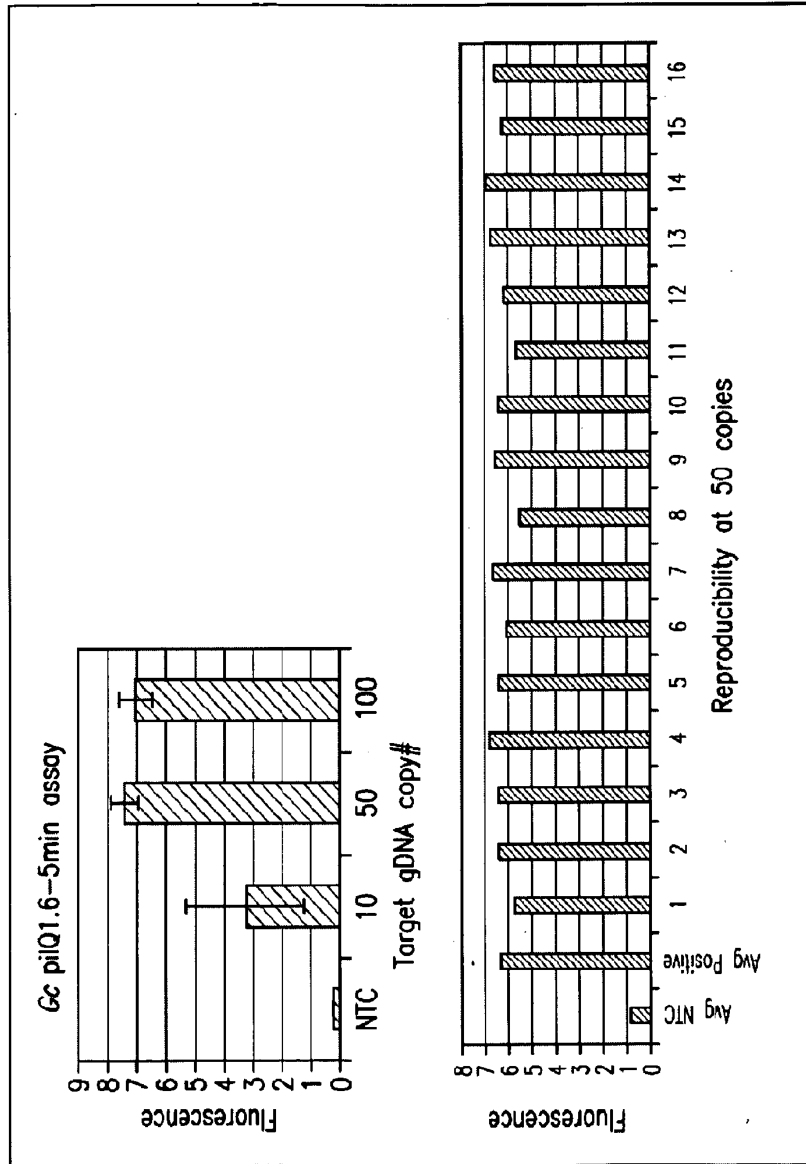


FIG.27

B. subtilis 1.25 NEAR Assay

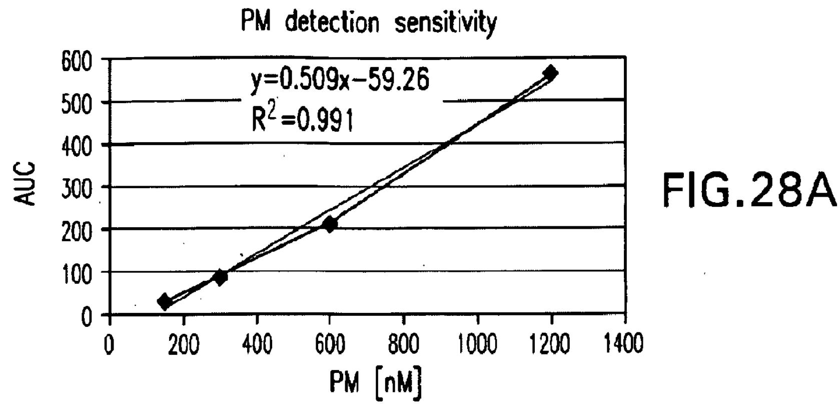


FIG.28A

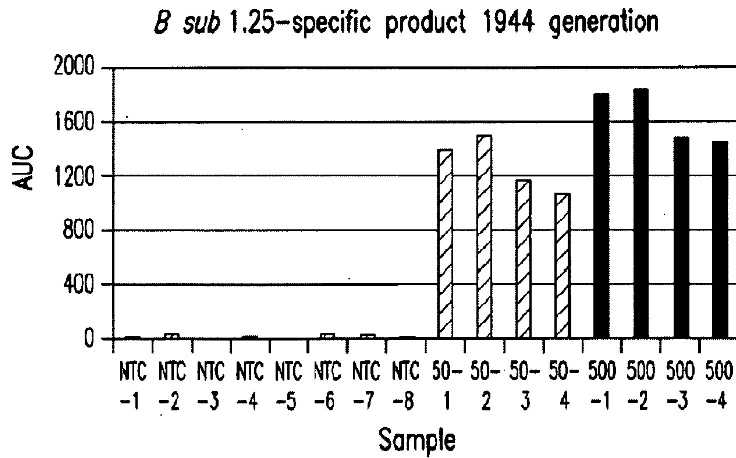


FIG.28B

Specific product 1944 yields (x=y-b/m)		
Sample	AUC signal	Product [nM]
50-1	1394	2851
50-2	1495	3049
50-3	1175	2421
50-4	1072	2219
500-1	1799	3645
500-2	1837	3720
500-3	1472	3004
500-4	1438	2937

FIG.28C

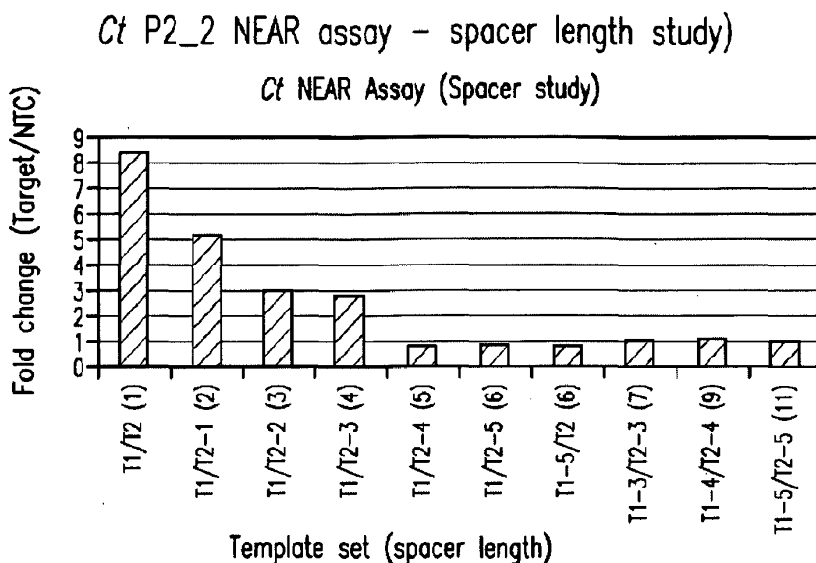
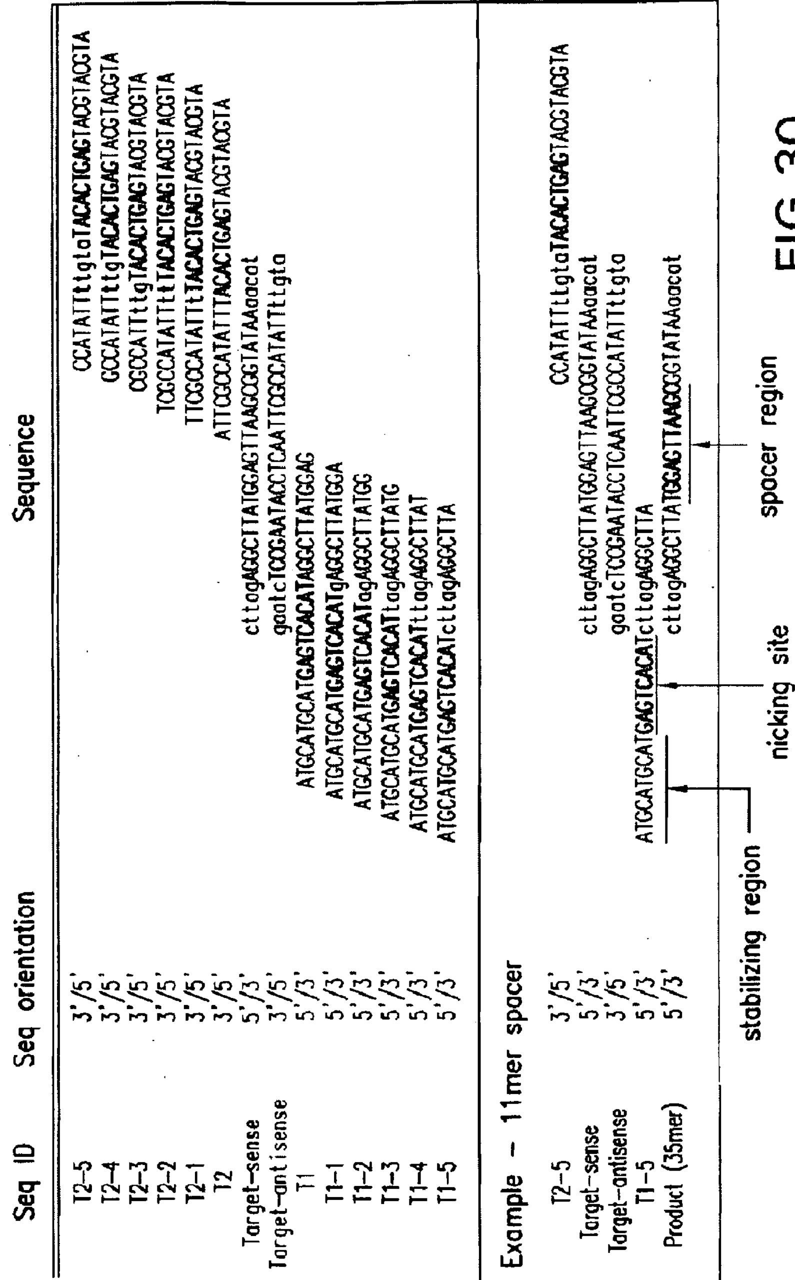


FIG. 29A

Temp1	12mer	ATGCATGCATGAGTCACATAGGCTTATGGAG
Temp1	-1 12mer	ATGCATGCATGAGTCACATgAGGCTTATGGA
Temp1	-2 12mer	ATGCATGCATGAGTCACATagAGGCTTATGG
Temp1	-3 12mer	ATGCATGCATGAGTCACATtagAGGCTTATG
Temp1	-4 12mer	ATGCATGCATGAGTCACATttagAGGCTTAT
Temp1	-5 12mer	ATGCATGCATGAGTCACATcttagAGGCTTA
Temp2	12mer	ATGCATGCATGAGTCACATTTATACCGCTTA
Temp2	-1 12mer	ATGCATGCATGAGTCACATtTTATACCGCTT
Temp2	-2 12mer	ATGCATGCATGAGTCACATttTTATACCGCT
Temp2	-3 12mer	ATGCATGCATGAGTCACATgttTTATACCGC
Temp2	-4 12mer	ATGCATGCATGAGTCACATtgttTTATACCG
Temp2	-5 12mer	ATGCATGCATGAGTCACATatgttTTATACC

FIG. 29B


Ct NEAR assay-Template designs to analyze effect of spacer length

Seq ID	Seq orientation	Sequence
T2-5	3'/5'	CCATATTttgtaTACACTGAGTACGTACGTA
T2-4	3'/5'	GCCATATTttgTACACTGAGTACGTACGTA
T2-3	3'/5'	CGCCATTttgTACACTGAGTACGTACGTA
T2-2	3'/5'	TCGCCATATTttTACACTGAGTACGTACGTA
T2-1	3'/5'	TTCGCCATATTtTACACTGAGTACGTACGTA
T2	3'/5'	ATTCGCCATATTTACACTGAGTACGTACGTA
Target-sense	5'/3'	cttagAGGCTTATGGAGTTAAGCGGTATAAaacat
Target-antisense	3'/5'	gaatcTCCGAATACCTCAATTCGCCATATTttgta
T1	5'/3'	ATGCATGCATGAGTCACATAGGCTTATGGAG
T1-1	5'/3'	ATGCATGCATGAGTCACATgAGGCTTATGGA
T1-2	5'/3'	ATGCATGCATGAGTCACATagAGGCTTATGG
T1-3	5'/3'	ATGCATGCATGAGTCACATtagAGGCTTATG
T1-4	5'/3'	ATGCATGCATGAGTCACATttagAGGCTTAT
T1-5	5'/3'	ATGCATGCATGAGTCACATcttagAGGCTTA

Example - 11mer spacer

Seq ID	Seq orientation	Sequence
T2-5	3'/5'	CCATATTttgtaTACACTGAGTACGTACGTA
Target-sense	5'/3'	cttagAGGCTTATGGAGTTAAGCGGTATAAaacat
Target-antisense	3'/5'	gaatcTCCGAATACCTCAATTCGCCATATTttgta
T1-5	5'/3'	ATGCATGCATGAGTCACATcttagAGGCTTA
Product (35mer)	5'/3'	cttagAGGCTTATGGAGTTAAGCGGTATAAaacat

FIG.30

Ct NEAR assay- requirement for template 'Stabilizing region'

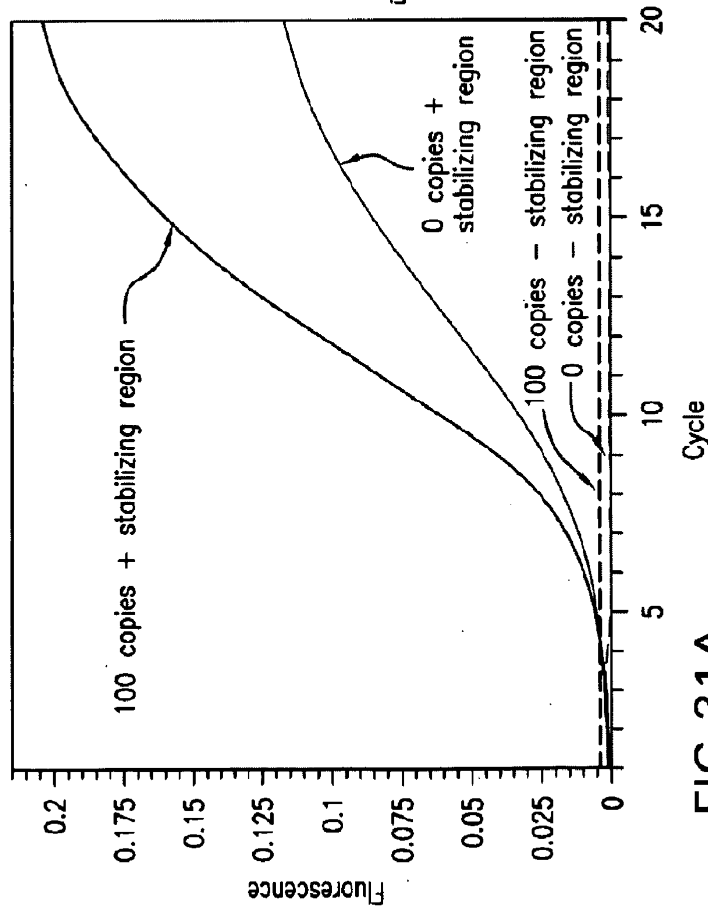


FIG.31A

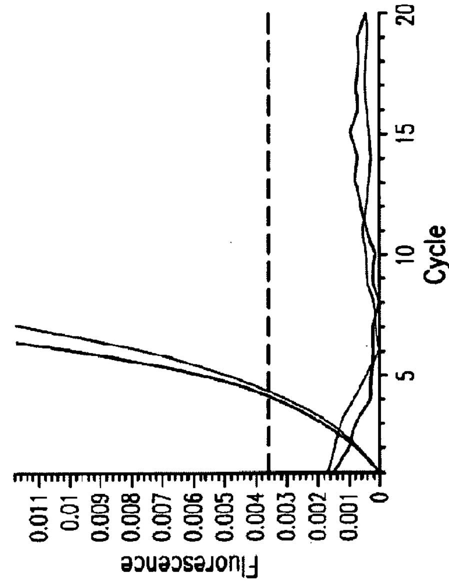


FIG.31B

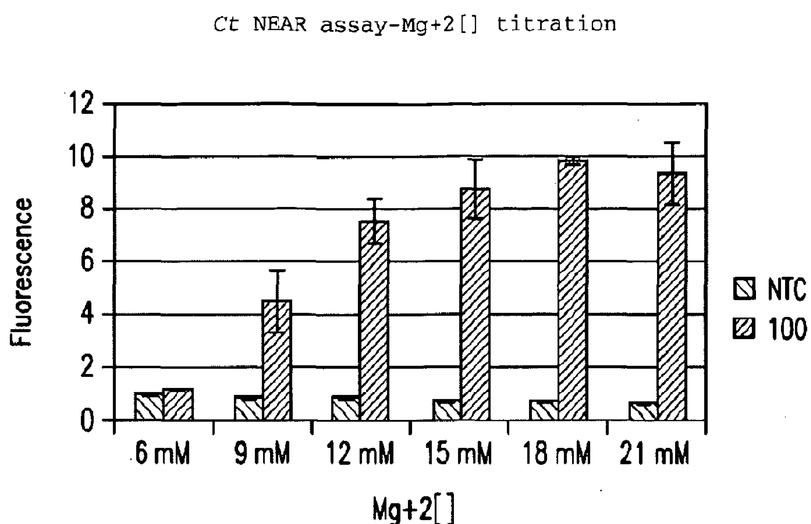


FIG.32A

Assay ID	*Ct* Ps_2	
Target	*Chlamydia trachomatis*	
	synthetic	
MB ID/[nM]	MB5.18/400	
Template ratio [nM]	200:100	
Replicates	2	
Experiment Date	1/14/2008	
Step	Time (min)	Temp (°C)
Reaction	5	56
Enzyme Inactivation	2	80
Readout	1	56
Comments: Comments: In-house buffer was used at 50mM Tris-HCl, pH8.6, 1mM DTT, 0.1% Tx-100		

FIG.32B

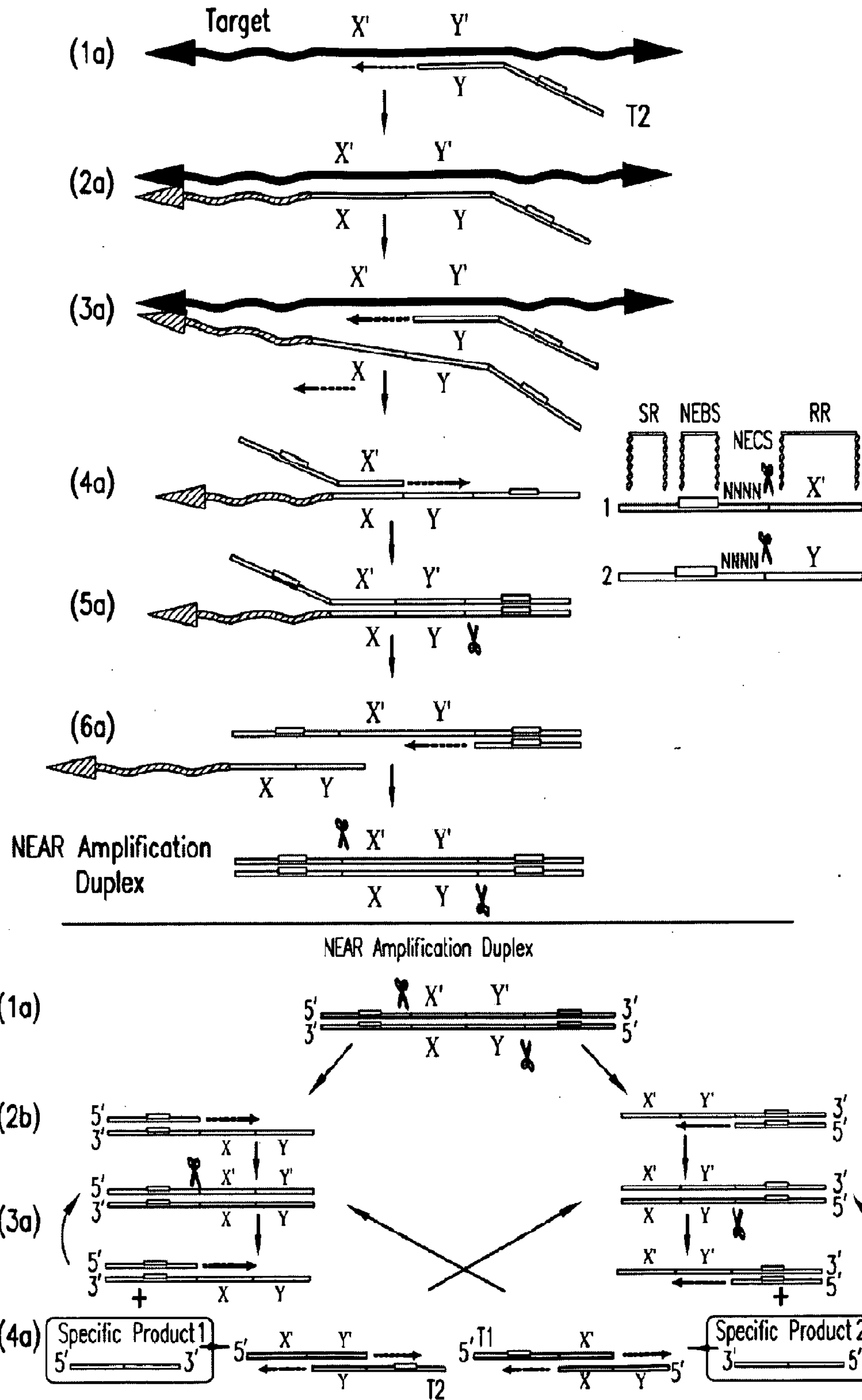


FIG.33

Target Organism	Target	Genome	Assay
Chlamydia trachomatis	Bacteria	DNA	P2_2
C trachomatis	Bacteria	RNA	P2_2
Neisseria gonorrhoeae	Bacteria	DNA	16S-4
N gonorrhoeae	Bacteria	DNA	pilQ 1.6
Mycobacterium tuberculosis	Bacteria	DNA	ITS.23s.5s.A.F/R1.12
Enterovirus	Virus	RNA	F8/R9.11
Clostridium difficile	Bacteria	DNA	TcdB F24/R25
C difficile	Bacteria	DNA	TcdB F25/R25
C difficile	Bacteria	DNA	TcdB F24/R24
Listeria monocytogenes	Bacteria	DNA	Lmono 579
Foot & Mouth disease virus	Virus	RNA	F1.2/R1.2
Foot & Mouth disease virus	Virus	RNA	F1.7/R1.7
Human miRNA	Eukaryote	RNA	miRNA 21
Human miRNA	Eukaryote	RNA	miRNA 335
Bacillus subtilis	Bacteria	DNA	ppsA 1.25
B subtilis	Bacteria	RNA	ppsA 1.25
Adenovirus 5	Virus	DNA	E1A 1.11
Methicillin-resistant Staphylococcus aureus	Bacteria	DNA	mecA 1359
MRSA	Bacteria	DNA	mecA 1520
MRSA	Bacteria	DNA	SA_nuc 355
MRSA	Bacteria	DNA	SA_nuc 368
MRSA	Bacteria	DNA	SA_nuc 662
Salmonella spp	Bacteria	DNA	spaO 4
Acinetobacter baumanii	Bacteria	DNA	A.ba.gyrB.A.12.F9/R9
Escherichia coli	Bacteria	DNA	Ecoli 4.F/R

FIG.34A

Template (5'-3')	Template 2 (5'-3')	Target (5'-3')
ATGCATGCATGAGTCACATAGGCTTATGGAG	ATGCATGCATGAGTCACATTTATACCGCTTA	AGGCTTATGGAGTTAAGCGGTATAA
ATGCATGCATGAGTCACATAGGCTTATGGAG	ATGCATGCATGAGTCACATTTATACCGCTTA	AGGCTTATGGAGTTAAGCGGTATAA
ATGCATGCATGAGTCACATCGCATACGTCTT	ATGCATGCATGAGTCACATCCTGCTTTCCCT	CGCATACGTCTTGAGAGGGAAAGCAGG
ATGCATGCATGAGTCACATTTTTTGAGTTCC	ATGCATGCATGAGTCACATACTCTACCAACA	ACTCTACCAACACGGAACTCAAAAA
ATGCATGCATGAGTCACATAAACAAACTCGC	ATGCATGCATGAGTCACATAACGGATGTGGT	AAACAAACTCGCAACCACATCCGTT
ATGCATGCATGAGTCACATCGACTACTTTGG	ATGCATGCATGAGTCACATGAAACACGGAC	CGACTACTTTGGGTGTCCGTGTTTC
ATGCATGCATGAGTCACATAGAAACTGGAGA	ATGCATGCATGAGTCACATCTACAAATATAG	AGAAACTGGAGAATCTATATTTGTAG
ATGCATGCATGAGTCACATGAAACTGGAGAA	ATGCATGCATGAGTCACATCTACAAATATAG	GAAACTGGAGAATCTATATTTGTAG
ATGCATGCATGAGTCACATAGAAACTGGAG	ATGCATGCATGAGTCACATCAAATATAGAT	AGAAACTGGAGAATCTATATTTG
ATGCATGCATGAGTCACATAAAGCAAGAGAA	ATGCATGCATGAGTCACATATACACGATAAC	AAAGCAAGAGAAAGTTATCGTGTAT
ATGCATGCATGAGTCACATAGGCTAAGGATG	ATGCATGCATGAGTCACATGGTACCTGAAGG	AGGCTAAGGATGCCCTTCAGGTACC
ATGCATGCATGAGTCACATGCCCTTCAGGTA	ATGCATGCATGAGTCACATTGTTACCTCGGG	GCCCTTCAGGTACCCCGAGGTAACA
ATGCATGCATGAGTCACATTAGCTTATCA	ATGCATGCATGAGTCACATTCAACATCAG	UAGCUUAUCAGACUGAUGUUGA
ATGCATGCATGAGTCACATTCAAGAGCAA	ATGCATGCATGAGTCACATACATTTTTCGT	UCAAGAGCAAUAACGAAAAAUGU
ATGCATGCATGAGTCACATCCAAGCTCAAAA	ATGCATGCATGAGTCACATTTCACGATTCCT	CCAAGCTCAAAAAAGGAATCGTGAA
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ATGCATGCATGAGTCACATGATACACCTGAA	ATGCATGCATGAGTCACATTAGGATGCTTTG	GATACACCTGAAACAAAGCATCCTA
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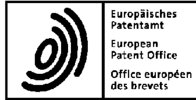
ATGCATGCATGAGTCACATGAAATGACCTAA	ATGCATGCATGAGTCACATCTACGCGAAAAA	GAAATGACCTAACTTTTTCGCGTAG
ATGCATGCATGAGTCACATAAATTCTCGTCT	ATGCATGCATGAGTCACATTTTCTTTTGTCT	AAATTCTCGTCTCAGACAAAAGAAA
ATGCATGCATGAGTCACATAGTTTCGACTGT	ATGCATGCATGAGTCACATGTTAAGCAGGAA	AGTTTCGACTGTTTTCCTGCTTAAC

FIG.34B



EUROPEAN SEARCH REPORT

Application Number
EP 12 19 5333

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The present search report has been drawn up for all claims

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Place of search	Date of completion of the search	Examiner
The Hague	27 September 2013	Aguilera, Miguel

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INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(51) International Patent Classification 5 :
C12Q 1/68, 1/70

A1

(11) International Publication Number: **WO 94/03635**

(43) International Publication Date: 17 February 1994 (17.02.94)

(21) International Application Number: PCT/GR92/00015

(22) International Filing Date: 4 August 1992 (04.08.92)

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(81) Designated States: JP, European patent (AT, BE, CH, DE, DK, ES, FR, GB, GR, IE, IT, LU, MC, NL, SE).

Published
With international search report.

(54) Title: RAPID AMPLIFICATION AND DETECTION OF NUCLEIC ACIDS

(57) Abstract

Disclosed herein are methods, primers, probes, and kits for the rapid amplification and detection of nucleic acids. In a preferred embodiment, a target nucleic acid sequence in a sample is amplified by: 1) adding nucleoside triphosphates, primer pairs comprising two oligonucleotide primers, and a nucleic acid polymerase to the sample; 2) denaturing the target nucleic acid sequence to form separate strands; and 3) maintaining a reaction temperature in a range from 68 °C to 80 °C and appropriate reaction conditions wherein the following cycle occurs: the primers hybridize to the target strands, primer extension products are formed, the extension products separate to become templates for the primers, and new extension products are formed. In an alternative embodiment, the amplification occurs at two different temperature ranges. Primer extension products are formed in a temperature range from 68 °C to 82 °C, and the products are separated by raising the temperature to range of 88 °C to 96 °C. Detection of the amplified sequences occurs by using some biotin-labeled nucleoside triphosphates, which produces nucleotide sequences that are copies of the target sequence and that contain one or more biotin-labeled nucleotides. The amplified sequences are detected by contacting the sample with immobilized probes and then detecting the presence of the biotin. A two-stage amplification process is also provided wherein: 1) the probe-target sequence complexes are contacted with a first moiety that binds to biotin; 2) a second moiety, comprising biotin bound to a detectable moiety is added, wherein the biotin in the second moiety binds to the first moiety; and 3) detecting or measuring the detectable moiety. In a particularly preferred embodiment, primer pairs and a probe are disclosed for use when the target nucleic acid is HIV-1 DNA.

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RAPID AMPLIFICATION AND DETECTION OF NUCLEIC ACIDS

FIELD OF THE INVENTION

This invention relates to the rapid amplification and detection of nucleic acids. In particular, the invention provides improved methods for amplifying small amounts of nucleic acids in a sample in which the amplifcation steps are conducted at the same temperature or, alternatively, at only two different temperatures. In addition, the invention provides improved methods for detecting the amplified nucleic acids in which the detection signal is boosted. Related probes and test kits are also provided. The invention is expected to be useful in a wide variety of fields, including scientific, clinical, and forensic analysis. In one specific embodiment, it is particularly useful in the detection of human immunodeficiency virus type 1 (HIV-1), the causative agent of AIDS.

BACKGROUND OF THE INVENTION

The ability to detect exceeding small amounts of a nucleic acid in a sample generally requires the amplification of the amount of the target nucleic acid. This is especially important for the detection of human retroviruses, where positive samples may contain only 5-10 target molecules in 10^6 cells.

The preferred method for amplifying target DNA has been the polymerase chain reaction (PCR) technique. The technique has been described in U.S. Patent Nos. 4,683,195, issued July 28, 1987 to Mullis, et al., 4,683,202, issued

July 28, 1987 to Mullis, 4,800,159, issued January 24, 1989 to Mullis, et al., 4,889,818, issued December 26, 1989 to Gelfand, et al., and 4,902,624, issued February 20, 1990 to Columbus, et al., all of which are incorporated herein by
5 reference.

In general, the PCR reaction involves the use of a pair of specific oligonucleotide primers to initiate DNA synthesis on a target DNA template. Two oligonucleotide primers are used for each double-stranded sequence to be
10 amplified. The target sequence is denatured into its complementary strands. Each of the primers, which are sufficiently complementary to a portion of each strand of the target sequence to hybridize with it, anneals to one of the strands. The primers are extended, using nucleosides
15 in the sample and a polymerization agent, such as heat-stable Taq DNA polymerase. This results in the formation of complementary primer extension products, which are hybridized to the complementary strands of the target sequence. The primer extension products are then separated
20 from the template strands, and the process is repeated until the desired level of amplification is obtained. In subsequent cycles, the primer extension products serve as new templates for synthesizing the desired nucleic acid sequence.

25 By repeating the cycles of denaturation, annealing, and extension, the original target DNA can be amplified exponentially according to the formula 2^n, where n is the number of cycles. In theory, 25 cycles, for example, would result in a 3.4×10^7-fold amplification. However, since
30 the efficiency of each cycle is less than 100%, the actual amplification after 25 cycles is about $1\text{-}3 \times 10^6$-fold. The

size of the amplified region is generally about 100-400 base pairs, although stretches of up to 2 kb can be amplified. See Keller and Manak, DNA Probes (New York: Stockton Press, 1989), pgs. 215-216.

5 The three basic steps of the PCR reaction -- denaturation, annealing, and extension -- are driven and controlled by the temperature of the reaction mixture, with each step occurring at a different temperature. Somewhat different temperature ranges are disclosed for each of the 10 three steps in the above-referenced patents. However, as time passed, those skilled in the art have settled on fairly standard temperatures for each of the steps in the cycle. Thus, Gelfand, et al., discloses a denaturing temperature range of about 90°-105°C, preferably 90°-100°C, 15 an annealing temperature range of about 35°-65°C, preferably 37°-60°C, and an extension temperature range of about 40°-80°C, preferably 50°-75°C. For Taq polymerase, which is the overwhelmingly preferred polymerase for the PCR reation, Gelfand, et al., refers to an annealing 20 temperature range of about 45°-58°C and an extension temperature range of about 65°-75°C. Columbus, et al., which is directed to a temperature cycling cuvette for use in PCR, refers to temperature ranges of 92°-95°C for the denaturing step, 50°-60°C for the annealing step, 70°C for 25 the extension step. Keller and Manak, cited above, refer to a denaturation temperature of about 93°C, an annealing temperature of 37°-55°C, and a primer extension temperature of 70°C.

The PCR technique has been modified to permit the 30 amplification of viral RNA. See Murakawa, et al., DNA, 7:287-295 (1988), which is incorporated herein by

reference. The article discloses the amplification of sequences from HIV-1 RNA templates for the identification of HIV-1 in peripheral blood and tissue samples obtained from AIDS and ARC patients. Total nucleic acid is isolated from infected cells, and the DNA is digested with RNase-free DNase so that it does not contribute to the final PCR product. A cDNA copy of a target sequence of the viral RNA is synthesized, using the PCR primers and reverse transcriptase. The one primer complementary to the RNA serves to initiate cDNA synthesis.

Several different formats have been used for the detection of PCR products. Generally, a radioactive or nonradioactive labeled probe that is complementary to the target sequence is used. The hybridization of the probes to the amplified target sequence, and the subsequent detection of the labeled moiety results in the detection of the target sequence. Nonradioactive labeled probes are generally more desirable because they obviate the need for special handling procedures. However, they may not generate as intense a signal, or the signal may be obscurred by background "noise." Thus, there is a need for enhancing the intensity of the signal in such probes.

The PCR technique is a revolutionary one, and it is widely used. Nevertheless, it does have significant drawbacks. The most serious of these is nonspecific hybridization, which results in false positives. Avoiding nonspecific hybridization requires ultrapure reagents. Unfortunately, for most clinical and diagnostic applications, it is desirable to use "dirty" samples, which presents a major problem, unless time-consuming and expensive sample preparation is undertaken.

Another important drawback to the PCR technique is the time involved in amplification. Although the usual six hour time period for PCR is far superior to an alternative technique such as cloning, which can take days or weeks, it would still be desirable to cut amplification time by one-half to two-thirds.

The present invention overcomes these drawbacks of the PCR technique, and it provides an improved detection system. The invention provides methods for the rapid amplification and detection of nucleic acids in which the denaturing, annealing, and extension steps occur all at the same temperature or, alternatively, at only two different temperatures, thus providing for faster cycling. In addition, the invention provides amplification methods where the annealing temperature is higher than the prior art temperatures, thus eliminating nonspecific hybridization. Finally, the invention provides improved methods of detecting the amplified nucleic acids through a two-stage signal amplification.

SUMMARY OF THE INVENTION

It is an object of the present invention to provide methods for amplifying a target nucleic acid sequence in a sample.

Another object is to provide methods for detecting or measuring the presence of a target nucleic acid sequence in a sample.

Still another object of the invention is to provide a kit for detecting or measuring the presence of a target nucleic acid sequence in a sample.

A further object of the invention is to provide primer pairs for use in amplifying a nucleic acid sequence of HIV-1 DNA.

A still further object is to provide a probe for use in detecting a nucleotide sequence complementary to a target nucleic acid sequence of HIV-1 DNA.

Additional objects and advantages of the invention will be set forth in part in the description that follows, and in part will be obvious from the description, or may be learned by the practice of the invention. The objects and advantages of the invention will be attained by means of the instrumentalities and combinations particulary pointed out in the appended claims.

To achieve the objects and in accordance with the purpose of the invention, as embodied and broadly described herein, the present invention provides a method for amplifying a target nucleic acid sequence in a sample. The sequence is part of a nucleic acid, which the sample is suspected of containing. Preferably, the target nucleic acid is DNA, most preferably HIV-1 DNA.

Nucleoside triphosphates, primer pairs consisting of two oligonucleotide primers, and a nucleic acid polymerase are added to the sample. Each primer is an oligonucleotide having a region that is complementary to and hybridizes with a different strand of the target

sequence. It is effective as an initiator for nucleoside polymerization. If double stranded, the nucleic acid in the sample is denatured so that separate strands of the target nucleic acid sequence are formed. Preferably, the denaturing is accomplished by heating the sample to about 95°C.

A reaction temperature in a range from about 68°C to about 80°C (preferably about 75°C) and appropriate reaction conditions are maintained so that the following cycle occurs. First, the primers anneal (hybridize) to the separate strands of the target sequence. The primers and the nucleic acid polymerase initate the synthesis of primer extension products, formed by the nucleosides attaching to the primer and forming a polymer, using the target sequence strand as a template. Thus, the primer extension products are annealed or hybridized to the strands. The primer extension products are then separated from the strands to become templates for the primers, and the cycle is repeated, with new primer extension products being formed. The reaction is allowed to continue for a sufficient number of cycles until the desired amplification of the target nucleic acid sequence has been accomplished. The primers are chosen so that they bind strongly to the strands of the target sequence, but the extension from each primer binds weakly to the strand.

In an alternative embodiment of the invention, the cycling occurs at two different temperatures. The reaction temperature is maintained in a range from about 68°C to about 82°C (preferably about 70°C) and appropriate reaction conditions are maintained to form the primer extension products. These are separated from the strands by raising

the temperature to a range of about 88°C to about 96°C (preferably about 90°C) to produce single-stranded molecules. The temperature is then lowered back to the previously mentioned range, permitting the primers to anneal to the single-stranded molecules and primer extension products to be synthesized, using the single-stranded molecules as templates. The temperature cycling is repeated a sufficient number of times to obtain the desired amplification of the target nucleic acid sequence.

The invention also provides methods for detecting or measuring a target nucleic acid sequence in a sample, based upon the amplification methods discussed above. In these methods, some of the nucleoside triphosphates are biotin-labeled. This produces nucleotide sequences that are copies of the target sequence which contain one or more biotin-labeled nucleotides. Such sequences are rendered single-stranded and contacted with immobilized probes. Each of the probes is a single-stranded polynucleotide attached to a solid support and capable of hybridizing with one of the single-stranded nucleotide sequences. The probes are contacted with the sample for a sufficient time and under appropriate hybridizing conditions to permit the polynucleotides to hybridize with the single-stranded nucleotide sequences. This forms bound complexes comprised of both of these entities. The presence of the biotin in the bound complexes is then detected or measured. Preferably, the biotin is detected by contacting the bound complexes with a detectable moiety that binds to biotin, such as avidin-horseradish peroxidase, and then detecting the detectable moiety, i.e., the horseradish peroxidase.

In an alternative and prefered embodiment, the detecting step is accomplished by contacting the bound complexes with a first moiety that binds to biotin, such as avidin. A second moiety, comprising biotin bound to detectable moity, such as horseradish peroxidase, is then added. The biotin in this second moiety binds to the first moiety. The detectable moiety is then detected. This provides a two-stage amplification of the signal.

The invention further provides kits for detecting or measuring the presence of a target nucleic acid sequence in a sample. The kits contain biotin-labeled nucleoside triphosphates and a primer pair specific for the particular target sequence sought to be detected or measured. Preferably, the kit further comprises a probe that is specific for the target sequence. It may also contain a nucleic acid polymerase, reagents for detecting or measuring the biotin, denaturing reagents, and/or controls.

In a preferred embodiment of the kit, the target nucleic acid sequence is HIV-1 DNA. The primer pair comprises the nucleotide sequences

5′ GAAGGAGCCA CCCCACAAG 3′ (SEQ ID NO: 1)

3′ CCCCCCTGTA GTTCGTCGG 5′ (SEQ ID NO: 2)

and the probe comprises the nucleotide sequence

5′ TTTAAACACC ATGCTAAACA CAGT 3′. (SEQ ID NO: 3)

DETAILED DESCRIPTION OF THE INVENTION

Reference will now made in detailed to the presently preferred embodiments of the invention which, together with the following examples, serve to explain the 5 principles of the invention.

The invention provides improved methods for amplifying and detecting nucleic acids. The target nucleic acid may be single or double-stranded DNA or RNA from any organism. Such organisms include plants, animals, and 10 microorganisms, such viruses, viroids, mycoplasma, bacteria, and fungi. The viruses include DNA and RNA viruses. In a preferred embodiment, the viruses are retroviruses, and in a particularly preferred embodiment, they are the AIDS-related viruses (including HIV-1 and HIV-15 2). The term "animals" includes mammals, and, in a preferred embodiment, the target nucleic acid is mammalian nucleic acid, such as mitochondrial or genomic DNA or the various types of RNA. Such nucleic acid further includes human cellular oncogene sequences and human structural gene 20 sequences.

The sample may be anything that contains nucleic acid, obtained from a source by techniques known to those skilled in the art. It may be further processed by known techniques, such as being subjected to extraction 25 procedures, to render it in a form usable in the method of the invention. For example, the method of the present invention can be applied to determine if a patient has been infected by a virus. A sample of body fluid or tissue that contains the virus is obtained from the patient. In this 30 case, the sample should contain cells that are capable of

being infected by the virus or should contain body fluid in which the virus or viral nucleic acid is known to accumulate. The viral nucleic acid is extracted from the sample by known techniques.

5 A target nucleotide sequence within the target nucleic acid is selected so as to provide a target of sufficient specificity and uniqueness in order to be identified and distinguished from other nucleic acid molecules in the sample. Among other things, it will be 10 selected on the basis of a nucleotide base sequence specific to the organism. For many viruses, in particular those that mutate at high rates, it is advisable to select a target sequence from a well-conserved region of the viral genome.

15 The target nucleotide sequence may consist of from about 50 to about 4000 base pairs, but preferably consists of about 55 to about 200 base pairs, and most preferably consists of about 60 to about 100 base pairs. In the case of HIV-1 DNA, the preferred target sequences are the 20 nucleotide sequence 1317-1379 of pHXB2 and 331-530 of pHXB2, depending upon which primer pair of the invention is used to amplify the sequence.

In the preferred embodiment of the invention, the target sequence is amplified in a reaction that occurs at a 25 single temperature, after the initial denaturation of the nucleic acid, in contrast to the three different temperatures in the PCR technique. We call this method the Continuous Enzyme Reaction (CER). In the first step, certain reagents are added to the sample that contains the 30 target nucleic acid. In particular, nucleoside

triphosphates, including biotinylated nucleotide triphosphates, primer pairs, and a nucleic acid polymerase are added. Preferably, they are added as part of a buffered solution. Each primer is an oligonucleotide having a region that is complementary to and hybridizes with a different strand of the target sequence. It is effective as an initiator for nucleoside polymerization. Preferably, the length of each primer is approximately 15-25 nucleotides.

The target nucleic sequence, if it is double-stranded, is then separated into single strands by known techniques. Preferably, the nucleic acid is denatured by heating the sample to about 95°C. The target sequence may be denatured before the other reagents are added to the sample, although it is preferable to denature it afterwards.

The sample is then maintained under appropriate reaction conditions to permit the following cycle to occur: (1) each of the oligonucleotide primers anneals (hybridizes) to each of the strands of the denatured target sequence; (2) primer extension products are synthesized from the nucleoside triphosphates, with the systhesis being initiated by the primer and catalyzed by the nucleic acid polymerase; and (3) a portion of the extension products, which are hybridized to the strands of the target sequence, separate spontaneously from the strands. The separated extension products become templates for the primers, and the cycle is repeated. Such reaction conditions include maintaining the reaction temperature somewhere in the range from about 68°C to about 80°C. Preferably, the temperature is maintained at about 75°C.

The reaction is permitted to proceed for a sufficient period of time so that the cycle is repeated about 10 to about 25 times. This will occur over a time ranging from about 1.0 hours to about 2.5 hours. 5 Generally, the longer reaction time is preferred.

The nucleoside triphosphates are readily available commercially or can be synthesized by those skilled in the art. When the method of the invention is applied to the amplification of DNA, deoxyribonucleoside triphosphates are 10 used.

Most preferably, some of the nucleoside triphosphates or deoxyribonucleoside triphosphates are labeled with biotin. This permits the incorporation of a reporter molecule into the amplified sequences, which can 15 be detected by the means described below. Such biotin-labeled nucleoside triphosphates can be synthesized by known techniques or are commercially available. The preferred biotin-labeled nucleoside triphosphate is Bio-dUTP. The labeled nucleosides are used in a concentration 20 of about 10 uM to about 5mM, and preferably from about 0.1 mM to about 0.5 mM.

The polymerizing agent is any polymerase for nucleic acid. Preferably, it is Taq polymerase, which is heat stable under these reaction conditions.

25 The primer pairs consist of two oligonucleotide primers. Each primer is an oligonucleotide having a region that is complementary to and hybridizes with a different strand of the target sequence, and is effective as an

initiator (primer) for nucleoside polymerization. The primers of the invention are selected so as to meet two requirements. First, they must bind strongly to the strands of the target sequence to allow binding at a
5 temperature higher than the temperature preferred in the literature. Second, the product from the extension of the primers, which is synthesized from the nucleosides in the presence of the polymerase, is the least stable of a series of products which were initially selected and more likely
10 to dissociate spontaneously to single-stranded form, thus itself becoming a target sequence of the appropriate primer. As used herein, the term "bind strongly" and variations thereof means adequately stable single-stranded 16DNA-primer hybrids are formed at the high temperature of
15 the invention.

This is accomplished by choosing primers where the ratio of G and C to A and T in the primers ranges from about 1.5:1 to about 3.0:1. Preferably, the ratio is about 3.0:1. This is confirmed by the temperature of
20 dissociation (Td) of the primer pair and the melting temperature (Tm) of the extension products. The Td of the primer pair is calculated from the formula Td = 4°(C+G) + 2°(A+T). The Tm, which is the temperature at which the extension products are denatured, is calculated from the
25 formula:

$$Tm = 81.5^\circ + 16.6 \log Ci + 0.41 (\% C+G) - 0.72 (\% formamide) - 82^\circ/n - 1.5 (\% mismatch)$$

where Ci is the ionic strength of the reaction solution and n is the number of nucleotides.

For HIV-1 DNA, the following specific primers, which we call HOPE 1 and HOPE 2, are preferred:

HOPE 1: 5′ GAAGGAGCCA CCCCACAAG 3′ (SEQ ID NO: 1)

HOPE 2: 3′ CCCCCCTGTA GTTCGTCGG 5′ (SEQ ID NO: 2)

5 These primers produce HIV-1 DNA sequences in positions 1317-1379 of the plasmid pHXB2, which are 63 base pairs long. The Td for HOPE 1 is 62°C and that for HOPE 2 is 64°C. The Tm of the product, i.e., the primer extension, is 76.7°C.

20 The use of primers meeting the above-stated conditions permits the maintenance of temperature and other reaction conditions whereby the amplification cycle occurs at a single temperature rather than three different temperatures. In order for the reaction to occur 25 efficiently in terms of yielding double-stranded target DNA, the following reaction conditions should be considered:

1) The temperature should be high enough so that a substantial portion of the target DNA is found in single- 30 stranded form, available for primer hybridization (the activity of the polymerase is a limiting factor).

2) The concentration of the primers should be high enough so as to favor single-stranded DNA-primer hybrid formation.

3) The GC content of the primers should be such as to allow an adequately stable single-stranded DNA-primer hybrid to be formed at the high temperature chosen.

4) The primer extension product, on the contrary, should be as unstable as possible so that the probability for the two strands to dissociate spontaneously is as high as possible.

5) The polymerizing agent should be able to efficiently polymerize dNTPs at the high temperature chosen and also to incorporate the dNTP analogue used as a label onto DNA strands.

Generally, the cycle of primer annealing, primer extension, and extension product denaturing occurs from about 10 to about 25 times. The time period for this to occur is approximately 0.5 hours to overnight.

In an alternative and preferred embodiment, asymmetric concentrations of the primers are used. That is, the concentration of one of the primers exceeds that of the other by a factor of about 10^3 to 1, and preferably by about 10^2 to about 1. This permits the build up of only one instead of two primer extension products. Therefore, there is only a limited risk of DNA-DNA complexing during hybridization. Instead, the product DNA will bind to the primer or the probe that is used for detection of the amplified DNA. This enhances both the specificity and sensitivity of the assay.

When the nucleic acid to be amplified is RNA, certain modifications are made. The RNA template in infected cells is either viral mRNA or packaged virion RNA. Total nucleic acid is isolated in crude form by known 5 methods, or it can be purified by, for example, phenol-chloroform extraction. Residual DNA may be digested with RNase-free DNase so that it does not contribute to the amplified sequences. A cDNA copy of a target ribonucleotide sequence in the target RNA is synthesized, 10 using the primers and reverse transcriptase. The primer complementary to the target sequence serves to initate cDNA synthesis. The single-stranded cDNA product can be amplified after heat inactivation of the reverse transcriptase and adjustment of the reaction conditions to 15 those of the method of the invention.

In an alternative embodiment of the invention, the amplification of the target nucleic acid sequence occurs at two different temperatures. The primer annealing and extension steps occur at one temperature, and the 20 denaturing of the primer extension products occurs at another temperature. We call this method the Accelerated Chain Enzyme Reaction (ACER).

In particular, the reaction temperature is maintained at a point in the range from about 68°C to about 25 82°C for primer annealing and extension. Preferably, the reaction temperature for these steps of the cycle is about 70°C. The primer extension products are separated from the target sequence strands or other extension products by raising the temperature to a range of about 88°C to about 30 96°C. This denatures the extension products from the template, producing single-stranded molecules that serve as

further templates when the temperature is lowered. Preferably, the denaturing step occurs at a temperature of about 90°C.

The cycling between these two temperatures is
5 repeated about 10 to about 25 times. The reaction time for these steps is from about 0.5 hours to about 3 hours. Generally, the time is about 1.5 hours.

The preparation of the sample and the initial reaction mixture is essentially the same as in the single
10 temperature method. The target nucleic acid sequence is also denatured in the same manner. This method also lends itself to the use of asymmetric concentrations of primers and to the amplification of RNA as discussed previously.

The specific primers for any given target sequence
15 are determined as discussed above with respect to the single temperature embodiment. The primers will have the same general characteristics mentioned above. For HIV-1 DNA, the previously mentioned HOPE 1 and HOPE 2 primers are preferred. In addition, two other primers, which we
20 designate NIDA 1 and NIDA 2, are preferred. These primers comprise the following sequences:

NIDA 1: 5' GACATCGAGC TTGCTAGAAG 3' (SEQ ID NO: 4)

NIDA 2: 3' GGTGACGAAT TCGGAGTTAT 5' (SEQ ID NO: 5)

They produce HIV-1 DNA sequences in positions 331-530
25 $pHXB_2$.

In another embodiment, the invention comprises a method for detecting or measuring a target nucleic acid in a sample. The target sequence is amplified according to the methods discussed above, wherein some of the nucleoside
5 triphosphates are biotin-labeled. This produces nucleotide sequences that are copies of the target sequence and contain one or more biotin-labeled nucleotides. The amplified sequences, which will be double-stranded, are then rendered single-stranded by known techniques,
10 preferably heat denaturation at a temperature of about 88°C to about 96°C. The sample is then contacted with immobilized probes. These probes are single-stranded polynucleotides attached to a solid support. Each one is capable of hybridizing with one of the single-stranded
15 nucleotide sequences. The probes are contacted with the sample for a sufficient time and under appropriate hybridizing conditions known to those skilled in the art to permit the polynucleotides to hybridize with the single-stranded nucleotide sequences. This forms bound complexes
20 of the polynucleotides and the single-stranded nucleotide sequences.

The probes can be prepared by standard techniques known to those skilled in the art, given the particular target sequence and the teachings contained herein. When
25 the nucleic acid is HIV-1 DNA, the preferred probe comprises the sequence 5' TTTAAACACC ATGCTAAACA CAGT 3' (SEQ ID NO: 3), which we call the HOPE 3 probe. The HOPE 3 probe may also be used with a pyrimidine spacer at the 5'end. Preferably, the spacer is CTCTC, in which case we
30 call the probe HOPE 4.

The solid support may be any solid material to which the probe may be attached. Such material includes filters, resins, beads, cubes, and microtiter plates. Preferably, the solid supports are the wells in plastic
5 microtiter plates, such as polystyrene microtiter plates. Most preferably, the probe is first chemically coupled to a protein carrier, such as bovine serum albumin, which is then immobilized onto polystyrene microtiter plate wells. See Running and Urdea, BioTechniques, 8:276-277 (1990) and
10 Nagata, et al., FEBS Letters, 183:379-382 (1985), both of which are incorporated herein by reference.

The single-stranded polynucleotide of the probe is attached to the solid support covalently or noncovalently by means known to those skilled in the art. Preferably,
15 the probe is chemically coupled to a protein, such as bovine serum albumin, or other carrier, such as polyethylene glycol. A spacer between the carrier and the probe may also be used. The polynucleotide comprising the probe may contain from about 15 to about 2,000 nucleotides.
20 Preferably, it contains from about 15 to about 200 nucleotides and most preferably from about 15 to about 40 nucleotides.

The final step in this method of the invention is to detect or measure the presence of biotin in the bound
25 complex. One way of doing so is to contact the bound complexes with a detectable moiety that binds to the biotin and then to detect such moiety. Preferably, such detectable moiety is comprised of a molecule or compound that binds to the biotin, such as avidin or streptavidin,
30 coupled to a detectable entity (e.g., a detectable molecule or compound), such as horseradish peroxidase. The

horseradish peroxidase is detected by known means. Other detectable moieties/detectable entities are disclosed in U.S. patent No. 4,711,955 issued December 8, 1987 to Ward et al., which is incorporated herein by reference.

5 Preferably, the bound complexes are washed with a liquid, such as a buffer solution, before and/or after contact with the detectable moiety to remove any unbound, labeled sequences or labeled nucleoside triphosphates that may be present.

10 In a particulary preferred embodiment, the invention provides a method for a two-stage amplification of the signal provided by the biotin. The method may be used to detect target nucleic acid sequences that have been amplified through the formation of primer extension 15 products that contain one or more biotin-labeled nucleotides. Thus, it is particularly applicable to the amplification methods of the present invention, but it is not limited to them.

Such amplified sequences are rendered single-20 stranded, and the sample containing such single-stranded sequences is contacted with immobilized probes as discussed above. The bound complexes comprising the single-stranded sequences and the single-stranded polynucleotides of the probes are then contacted with a first moiety that binds to 25 biotin. The moiety is preferably avidin or streptavidin. A second moiety, comprising biotin bound to a detectable moiety, is then added to the sample. The biotin in the second moiety binds to the first moiety, which is bound to the biotin in the amplified sequences. The detectable 30 moiety is then detected or measured by standard techniques.

Preferably, such detectable moiety is horseradish peroxidase. Preferably, the solid support is washed between the steps discussed above in order to remove unbound materials that may interfere with the detection
5 procedure or give a false signal.

The present invention also comprises a kit for detecting or measuring the presence of a target nucleic acid sequence in a sample suspected of containing that sequence. The kit comprises biotin-labeled nucleoside
10 triphosphates and an appropriate primer pair. Each primer comprises an oligonucleotide having a region that is complementary to and hybridizes with a different strand of the target sequence and is effective as a primer for nucleoside polymerization. Preferably, the kit further
15 comprises a probe that is complementary to and hybridizes with the target sequence. Most preferably, the kit further comprises a nucleic acid polymerase, preferably Taq polymerase, and additional means, such as reagents, for detecting or measuring the biotin, denaturing double-
20 stranded polynucleotides, and providing a control against which the results may be evaluated.

In a particularly preferred embodiment, the invention provides a kit for detecting or measuring the presence of a target nucleic acid sequence in HIV-1 DNA in
25 a sample. The kit contains biotin-labeled nucleoside triphosphates, unlabeled nucleoside triphosphates, a primer pair comprising the nucleotide sequences:

5′ GAAGGAGCCA CCCCACAAG 3′ (SEQ ID NO: 1)

3′ CCCCCCTGTA GTTCGTCGG 5′ (SEQ ID NO: 2)

and a probe comprising the nucleotide sequence

5′ TTTAAACACC ATGCTAAACA CAGT 3′. (SEQ ID NO: 3)

It is to be understood that the application of the teachings of the present invention to a specific problem or environment will be within the capabilities of one having ordinary skill in the art in light of the teachings contained herein. Examples of the products and processes of the present invention appear in the following examples.

EXAMPLE 1

Isolation of DNA From Blood

For AIDS screening, the object is a rapid and safe extraction of DNA to allow the processing of many samples. The suggested method is the following. Four hundred microliters (400 ul) of blood is collected in a tube containing anti-clotting agent (citrate or EDTA) and is contrifuged (e.g. 3,000 g for 5 minutes or 10,000 g for 5-10 seconds). The upper buffy-coat layer is removed, or the whole pellet is suspended in 200 ul distilled water in order to lyse the cells. The lysed cell suspension is boiled at 100°C in order to break up the cell nuclei. Centrifugation for 5 minutes at 10,000 g removes insoluble debris. The resultant supernatant is used for subsequent manipulations.

EXAMPLE 2

One Temperature Reaction (CER)

Fifteen ul of DNA sample prepared as in Example 1 are added to 80 ul of a reaction buffer. The reaction buffer contains 0.3nmol of each primer (HOPE 1, HOPE 2), 10mM Tris-HCl, pH 8.4 (20°C), 50mM KCl, 2mM $MgCl_2$, 0.1% gelatin. 0.5mM dATP, dCTP, dGTP, 150 uM dTTP, 350 uM Bio-11-dUTP, and 0.1% BSA (nuclease free). The reaction mix is incubated at 95°C for five minutes. This is to initially denature the DNA. The mix is then allowed to cool down to room temperature, three units of the enzyme Vent polymerase (New England Biolabs) are added, and mineral oil is layered at the top. The reaction then takes place at 78°C for 3-4 hours.

EXAMPLE 3

Two Temperature Reaction (ACER)

Ten microliters of the DNA containing supernatant prepared as in Example 1 are used for this amplification reaction. This volume is mixed with 90 ul of reaction buffer. The reaction buffer comprises 100pmol each primer (melting temperature higher than 62°C, i.e., NIDA1, NIDA2), 10mM Tris-HCl, pH 8.0 (20°C), 1.8mM $MgCl_2$, 50 mM KCl, 0.05% gelatin, 0.1mM dATP, dCTP, dGTP, 03nM dTTP, 70nM Bio-11-dUTP, and 0.1% BSA (nuclease free).

The reaction mixture is then layered with one or two drops of any mineral oil and transferred to a programmable heat block where amplification takes place.

The temperatures used are 90°C for the initial and subsequent denaturation steps, and 70°C for annealing and elongation. The incubation period allowed for denaturation is one minute and for annealing, which is coupled with
5 elongation, two minutes. This thermal profile is repeated 20 times, with the annealing-elongation step of the 20th cycle being the last step of the reaction. Reaction products are stored at 4°C for further manipulation. The amplification reaction takes place in the wells of a 96-
10 well ELISA plate, a very convenient way when dealing with a large number of samples.

EXAMPLE 4

Two Temperature Asymmetric Reaction (AACER)

This type of reaction is essentially the same as
15 the ACER, except for the amount of the primers, which now is 20pmol for HOPE 1 and 300pmol for HOPE 2. The volume of the DNA sample is also increased to 20 ul.

EXAMPLE 5

Detection of Amplified Products

20 The totality (100 ul) of the amplification reaction volume (ACER or CER) is applied to a well containing immobilized probe after heat denaturation (100°C, five minutes) and snap cooling on ice. (Denaturation is not necessary when asymetric two temperatures, or one
25 temperature reactions are performed.) Hybridization is allowed to proceed for one hour at 37°C. Unbound material is removed by washing four times with 0.0M NaCl.

A 50ng/ml solution of avidin-HRP in 10mM Tris-HCl pH 7.4 (20°C), 5% gelatin, and 2% BSA is added to the well. After incubating 30 minutes at room temperature with shaking, the well is washed with 0.9M NaCl, 10mM Tris-HCl, 5 pH 7.4 (20°C), and 0.1% Tween-20. The substrate mix is then applied (0.05% TetraMethylBenzidine, 10% H_2O_2). Blue color develops within 10 minutes. The colorigenic reaction is stopped with 0.5% H_2SO_4, and absorbence at 450nm is measured.

10 EXAMPLE 6

Preparation of the Primers

The synthesis of the primers is standard and automated (Applied Biosystems synthesizer, model 380B). Synthesis is based on a solid phase chemistry where the 15 first base is already bound to a controlled pore glass (CPG) support at the 3' end. The 5' end is protected with a dimethoxytrityl group. Each new base is covalently coupled to the previous base at the 5' end using a complete synthesis cycle. The cycle consists of 4 steps:

20 1. Detritylation. The attached (terminal) base is detritylated with trichloroacetic acid creating a free hydroxyl group.

2. Activation and addition. The next base is activated with tetrazole at the 3' end and then is passed 25 through the CPG column allowing it to react with 5' hydroxyl of the terminal base.

3. Capping. The unreacted 5' hydroxyl groups (approximately 0-2%) are blocked with acetic anhydride (acetylation).

4. Oxidation. The phosphorous of the new base is
5 oxidized from the trivalent to the pentavalent form using a iodine-water-lutidine-tetrahydofuran mixture.

After the completion of the synthesis cycle, the new base is accessible for the repeat of the cycle with the next base. Thus, elongation of the DNA chain is achieved
10 with the correct nucleotide sequence.

At the end of the run, the product is cleaved from the CPG using 25% ammonia solution. The bases of the resultant oligonucleotide are then deprotected by heat-treatment at 55°C in the presence of ammonia for 5 hours.

15 The product is evaporated to dryness to remove the ammonia, reconstituted in water and dried again, and finally reconstituted in water. The product is stored at -20°C until further use.

EXAMPLE 7

20 Preparation of the Probe

The oligonucleotide HOPE4 (5' CTCTCTTTAA ACACCATGCT AACACAGT 3') (SEQ ID NO:6) was synthesized using a DNA synthesizer (Applied Biosystems). It was then chemically coupled to bovine serum albumin, which was immobilized into
25 the wells of a polystyrene microtiter plate according to

the method of Running and Urdea, op. cit., and Nagata, et al., op. cit.

EXAMPLE 8

Evaluation of Samples

5 Eight seropositive samples were examined, plus a DNA sample prepared from an infected culture of CEM cells. Several seronegative samples were also examined. A positive control-plasmid derived cloned sequences of HIV-I, and a negative control, were always included.

10 Some samples were analyzed by applying CER and others by ACER. Amplification products were detected by either in solution hybridization with a specific radiolabeled probe (HOPE 3) and subsequent acrylamide gel electrophoresis or the ELISA-like colorimetric assay using 15 as label a biotinylated dNTP analog or a labeled primer (HOPE 2).

The values obtained were as follows:

Absorbance readings of ELISA-like assay:

	1 POS)	0.40	seropositive (5 yrs)
20	2 POS)	0.30	seropositive (1 yr)
	3 POS)	0.60	dead
	4 POS)	0.20	seropositive (1.5 yrs)
	5 POS)	0.40	under AZT (4 yrs)
	6 POS)	0.55	dead
25	7 POS)	0.30	seropositive (2 yrs)
	8 POS)	0.20	seropositive (1 yr)

9 CEM)	0.60	infected cell line
10 CONT)	1.00	plasmid clone of HIV-I
11 CONT)	0.06	yeast DNA
12 CONT)	0.05	no DNA
13 NEG)	0.07	seronegative
14 NEG)	0.06	seronegative

SEQUENCE LISTING

(1) GENERAL INFORMATION:

(i) APPLICANTS: Nectarios Tavernarakis, George Hatzidakis, and Elias Krambovitis

5

(ii) TITLE OF INVENTION: RAPID AMPLIFICATION AND DETECTION OF NUCLEIC ACIDS

(iii) NUMBER OF SEQUENCES: 5

10

(iv) CORRESPONDENCE ADDRESS:
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15

(E) COUNTRY: U.S.A.
(F) ZIP: 20037

(v) COMPUTER READABLE FORM:
(A) MEDIUM TYPE: Floppy disk
(B) COMPUTER: IBM PC compatible

20

(C) OPERATING SYSTEM: PC-DOS/MS-DOS
(D) SOFTWARE: ASCII

(vi) CURRENT APPLICATION DATA:
(A) APPLICATION NUMBER:
(B) FILING DATE:
(C) CLASSIFICATION:

5 (vii) PRIOR APPLICATION DATA:
(A) APPLICATION NUMBER:
(B) FILING DATE:

(viii) ATTORNEY/AGENT INFORMATION:
(A) NAME: Karny, Geoffrey M.
10 (B) REGISTRATION NUMBER: 31,382
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15 (2) INFORMATION FOR SEQ ID NO:1:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 19 nucleotides
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
20 (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:1:

GAAGGAGCCA CCCCACAAG 19

(3) INFORMATION FOR SEQ ID NO:2:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 19 nucleotides
(B) TYPE: nucleic acid
5 (C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA

(xi) SEQUENCE DESCRIPTION; SEQ ID NO:2:

CCCCCCTGTA GTTCGTCGG 19

10 (4) INFORMATION FOR SEQ ID NO:3:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 24 nucleotides
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
15 (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:3:

TTTAAACACC ATGCTAAACA CAGT 24

(5) INFORMATION FOR SEQ ID NO:4:

20 (i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 20 nucleotides
(B) TYPE: nucleic acid

(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULAR TYPE: DNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:4:

5

GACATCGAGC TTGCTAGAAG 20

(6) INFORMATION FOR SEQ ID NO:5:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 20 nucleotides
(B) TYPE: nucleic acid
10
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:5:

GGTGACGAAT TCGGAGTTAT 20

15 (7) INFORMATION FOR SEQ ID NO:6:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 28 nucleotides
(B) TYPE: nucleic acid
20
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:6:

CTCTCTTTAA ACACCATGCT AACACAGT 28

WE CLAIM:

1. A method for amplifying a target nucleic acid sequence in a sample, wherein said target sequence consists of two complementary strands, comprising the steps of:

(a) adding nucleoside triphosphates, primer
5 pairs comprising two oligonucleotide primers, and a nucleic acid polymerase to said sample;

(b) denaturing said target nucleic acid sequence to form separate strands; and

(c) maintaining a reaction temperature in a
10 range from about 68°C to about 80°C and appropriate reaction conditions wherein the following cycle occurs: said primers hybridize to said strands of said target sequence, primer extension products, which are hybridized to said strands, are formed from said primers and said
15 nucleoside triphosphates, said extension products separate from said strands to become templates for said primers, and new primer extension products are formed.

2. The method of claim 1 wherein said target sequence is denatured by heating said sample to about 95°C.

20 3. The method of claim 1 wherein the reaction temperature in step (c) is maintained at about 75°C.

4. The method of claim 1 wherein said primers bind strongly to the strands of said target sequence and the extensions from said primers bind weakly to the strands
25 of said target sequence.

5. The method of claim 4 wherein the ratio of G and C to A and T in said primers ranges from about 1.5:1 to about 3.0:1.

6. The method of claim 5 wherein said ratio is
5 about 3.0:1.

7. The method of claim 5 wherein the length of said primers is about 15-25 nucleotides.

8. The method of claim 5 wherein the concentration of one of said primers in said primer pair
10 exceeds the concentration of the other primer by a factor of about 10^3 to 1 to about 10^2 to 1.

9. The method of claim 1 wherein the cycle of step (c) is repeated about 10 to about 25 times.

10. The method of claim 1 wherein the reaction
15 temperature and conditions of step (c) are maintained for a time from about 1.0 hours to about 2.5 hours.

11. The method of claim 1 wherein said nucleic acid is AIDS-related viral nucleic acid.

12. The method of claim 1 wherein said target
20 nucleic acid is RNA and said sample is treated with reverse transcriptase.

13. The method of claim 1 wherein said target nucleic acid is DNA and said nucleoside triphosphates are deoxynucleoside triphosphates.

14. The method of claim 13 wherein said DNA is HIV-1 DNA.

15. The method of claim 14 wherein said target sequence is the nucleotide sequence 1317-1379 of pHXB2.

5 16. The method of claim 15 wherein said primer pair comprises the nucleotide sequences

5' GAAGGAGCCA CCCCACAAG 3' (SEQ ID NO: 1)

3' CCCCCCTGTA GTTCGTCGG 5'. (SEQ ID NO: 2)

17. A method for detecting or measuring a target
10 nucleic acid sequence in a sample, wherein said target sequence consists of two complementary strands, comprising the steps of:

(a) amplifying said target sequence according to claim 1, wherein some of said nucleoside triphosphates
15 are biotin-labeled, thereby producing nucleotide sequences that are copies of said target nucleotide sequence and contain one or more biotin-labeled nucleotides;

(b) rendering said nucleotide sequences single-stranded;

20 (c) contacting said sample containing said single-stranded nucleotide sequences with immobilized probes, each of which comprises a single-stranded polynucleotide attached to a solid support and capable of hybridizing with one of said single-stranded nucleotide

sequences, for a sufficient time and under appropriate hybridizing conditions to permit said polynucleotides to hybridize with said single-stranded nucleotide sequences, thereby forming bound complexes of said polynucleotides and
5 said single-stranded nucleotide sequences; and

(d) detecting or measuring the presence of biotin in said bound complexes.

18. The method of claim 17 wherein said solid support is a microtiter plate.

10 19. The method of claim 17 wherein said detecting or measuring step comprises the steps of:

contacting said bound complexes with a detectable moiety that binds to biotin; and

detecting or measuring said detectable moiety.

15 20. The method of claim 19 wherein said detectable moiety is avidin-horseradish peroxidase.

21. The method of claim 17 wherein said detecting or measuring step comprises the steps of:

contacting said bound complexes with a first
20 moiety that binds to biotin;

adding a second moiety comprising biotin bound to a detectable moiety, whereby the biotin in said second moiety binds to said first moiety; and

detecting or measuring said detectable moiety.

22. The method of claim 21 wherein said first moiety is avidin and said detectable moiety is horseradish peroxidase.

5 23. The method of claim 19 wherein said nucleic acid is HIV-1 DNA and said single-stranded polynucleotide of said probe comprises the sequence

5′ TTTAAACACC ATGCTAAACA CAGT 3′ (SEQ ID NO: 3) or

5′ CTCTCTTTAA ACACCATGCT AACACAGT 3′ (SEQ ID NO: 6).

10 24. A method for amplifying a target nucleic acid sequence in a sample, wherein said target sequence consists of two complementary strands, comprising the steps of:

(a) adding nucleoside triphosphates, primer pairs comprising two oligonucleotide primers, and a nucleic
15 acid polymerase to said sample;

(b) denaturing said target nucleic acid sequence to form separate strands;

(c) maintaining the reaction temperature in a range from about 68°C to about 82°C and appropriate
20 reaction conditions to form primer extension products from said primers and said nucleoside triphosphates, wherein said extension products are hybridized to said strands;

(d) separating said extension products from said strands by raising the temperature to a range of about 88°C to about 96°C to produce single-stranded molecules;

(e) lowering the reaction temperature to a range from about 68°C to about 82°C and maintaining appropriate reaction conditions to synthesize primer extension products from said primers and said nucleoside triphosphates using the single-stranded molecules produced by step (d) as a template; and

(f) repeating steps (d) and (e) a sufficient number of times to obtain the desired amplification of said target nucleic acid sequence.

25. The method of claim 24 wherein the temperature at which step (d) is performed is about 90°C and the temperature at which steps (c) and (e) is performed is about 70°C.

26. The method of claim 24 wherein said primers bind strongly to the strands of said target sequence and the extensions from said primers bind weakly to the strands of said target sequence.

27. The method of claim 26 wherein the ratio of G and C to A and T in said primers ranges from about 1.5:1 to about 3.0:1.

28. The method of claim 24 wherein the reaction time for steps (c)-(f) is from about 0.5 hours to about 3 hours.

29. The method of claim 24 wherein said nucleic acid is HIV-1 DNA and said nucleoside triphosphates are deoxynucleoside triphosphates.

30. The method of claim 29 wherein said target
5 sequence is the nucleotide sequence 1317-1379 of pHXB2.

31. The method of claim 30 wherein said primer pair comprises the nucleotide sequences

5′ GAAGGAGCCA CCCCACAAG 3′ (SEQ ID NO: 1)

3′ CCCCCCTGTA GTTCGTCGG 5′. (SEQ ID NO: 2)

10 32. The method of claim 29 wherein said target sequence is the nucleotide sequence 331-530 of pHXB2.

33. The method of claim 32 wherein said primer pair comprises the nucleotide sequences

5′ GACATCGAGC TTGCTAGAAG 3′ (SEQ ID NO: 4)

15 3′ GGTGACGAAT TCGGAGTTAT 5′. (SEQ ID NO: 5)

34. A method for detecting or measuring a target nucleic acid sequence in a sample, wherein said target sequence consists of two complementary strands, comprising the steps of:

20 (a) amplifying said target sequence according to claim 25, wherein some of said nucleoside triphosphates are biotin-labeled, thereby producing nucleotide sequences

that are copies of said target nucleotide sequence and contain one or more biotin-labeled nucleotides;

(b) rendering said nucleotide sequences single-stranded;

5 (c) contacting said sample containing said single-stranded nucleotide sequences with immobilized probes, each of which comprises a single-stranded polynucleotide attached to a solid support and capable of hybridizing with one of said single-stranded nucleotide 10 sequences, for a sufficient time and under appropriate hybridizing conditions to permit said polynucleotides to hybridize with said single-stranded nucleotide sequences, thereby forming bound complexes of said polynucleotides and said single-stranded nucleotide sequences; and

15 (d) detecting or measuring the presence of biotin in said bound complexes.

35. The method of claim 34 wherein said detecting or measuring step comprises the steps of:

contacting said bound complexes with a 20 detectable moiety that binds to biotin; and

detecting or measuring said detectable moiety.

36. The method of claim 34 wherein said nucleic acid is HIV-1 DNA and said single-stranded polynucleotide of said probe comprises the sequence

25 5′ TTTAAACACC ATGCTAAACA CAGT 3′. (SEQ ID NO: 3)

37. A method for detecting or measuring the presence of a target nucleic acid sequence in a sample comprising the steps of:

(a) amplifying said target nucleic acid
5 sequence through the formation of primer extension products that contain one or more biotin-labeled nucleotides;

(b) rendering said primer extension products single-stranded;

(c) contacting said sample containing said
10 single-stranded sequences with immobilized probes, each of which comprises a single-stranded polynucleotide attached to a solid support and capable of hybridizing with one of said single-stranded nucleotide sequences, for a sufficient time and under appropriate hybridizing conditions to permit
15 said polynucleotides to hybridize with said single-stranded nucleotide sequences, thereby forming bound complexes of said polynucleotides and said single-stranded nucleotide sequences;

(d) contacting said bound complexes with a
20 first moiety that binds to biotin;

(e) adding a second moiety, comprising biotin bound to a detectable moiety, wherein the biotin in said second moiety binds to said first moiety; and

(f) detecting or measuring said detectable
25 moiety.

38. The method of claim 37 wherein said first moiety is avidin and said detectable moiety is horseradish peroxidase.

39. The method of claim 37 wherein said amplifying step is performed according to claim 1.

40. The method of claim 37 wherein said amplifying step is performed according to claim 24.

41. A primer pair for use in amplifying a target nucleic acid sequence of HIV-1 DNA comprising the nucleotide sequences

5′ GAAGGAGCCA CCCCACAAG 3′ (SEQ ID NO: 1)

3′ CCCCCCTGTA GTTCGTCGG 5′. (SEQ ID NO: 2)

42. A primer pair for use in amplifying a target nucleic acid sequence of HIV-1 DNA comprising the nucleotide sequences

5′ GACATCGAGC TTGCTAGAAG 3′ (SEQ ID NO: 4)

3′ GGTGACGAAT TCGGAGTTAT 5′. (SEQ ID NO: 5)

43. A probe for use in detecting a nucleotide sequence complementary to a target nucleic acid sequence of HIV-1 DNA, said probe comprising the nucleotide sequence

5′ TTTAAACACC ATGCTAAACA CAGT 3′ (SEQ ID NO: 3) or

5′ CTCTCTTTAA ACACCATGCT AACACAGT 3′ (SEQ ID NO: 6).

44. A kit for detecting or measuring the presence of a target nucleic acid sequence in a sample suspected of containing said sequence comprising, in a container:

5 (a) biotin-labeled nucleoside triphosphates; and

(b) a primer pair consisting of two oligonucleotide primers, each primer comprising an oligonucleotide having a region that is complementary to
10 and hybridizes with a different strand of said target sequence and being effective as a primer for nucleoside polymerization.

45. The kit of claim 44 further comprising a probe that is complementary to and hybridizes with said target
15 sequence.

46. The kit of claim 45 further comprising means for detecting or measuring said biotin.

47. The kit of claim 46 further comprising a nucleic acid polymerase.

20 48. The kit of claim 44 wherein said primer pair comprises the nucleotide sequences

5′ GAAGGAGCCA CCCCACAAG 3′ (SEQ ID NO: 1)

3′ CCCCCCTGTA GTTCGTCGG 5′. (SEQ ID NO: 2)

49. The kit of claim 44 wherein said primer pair comprises the nucleotide sequences

5′ GACATCGAGC TTGCTAGAAG 3′ (SEQ ID NO: 4)

3′ GGTGACGAAT TCGGAGTTAT 5′. (SEQ ID NO: 5)

5 50. The kit of claim 45 wherein said probe comprises the nucleotide sequence

5′ TTTAAACACC ATGCTAAACA CAGT 3′. (SEQ ID NO: 3)

51. A kit for detecting or measuring the presence of a target nucleic acid sequence in HIV-1 DNA in a sample
10 suspected of containing said sequence comprising, in a container:

(a) biotin-labeled nucleoside triphosphates;

(b) a primer pair comprising the nucleotide sequences

15 5′ GAAGGAGCCA CCCCACAAG 3′ (SEQ ID NO: 1)

3′ CCCCCCTGTA GTTCGTCGG 5′ (SEQ ID NO: 2)

and

(c) a probe comprising the nucleotide sequence

20 5′ TTTAAACACC ATGCTAAACA CAGT 3′. (SEQ ID NO: 3)

INTERNATIONAL SEARCH REPORT

International application No.
F '/GR 92/00015

A. CLASSIFICATION OF SUBJECT MATTER

IPC5: C12Q 1/68, C12Q 1/70

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

IPC5: C12Q

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

MEDLINE, BIOSIS

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	THE JOURNAL OF INFECTIOUS DISEASES, Volume 161, No, 1990, Kathleen D. Eisenach et al, "Polymerase Chain Reaction Amplification of a Repetitive DNA Sequence Specific for Mycobacterium tuberculosis", see especially p 978, col. 1, lines 1-10 --	1-14,17-22, 24-30,34-35, 37-40,44-47
X	J CLIN PATHOL, Volume 45, No, 1992, E S-F Lo et al, "Detection of hepatitis B pre-core mutant by allele specific polymerase chain reaction", see especially p. 690 --	1-14,17-22, 24-30,34-35, 37-40,44-47

[X] Further documents are listed in the continuation of Box C. [X] See patent family annex.

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Date of the actual completion of the international search	Date of mailing of the international search report
10 February 1993	25.02.93
Name and mailing address of the ISA/ European Patent Office, P.B. 5818 Patentlaan 2 NL-2280 HV Rijswijk Tel. (+31-70) 340-2040, Tx. 31 651 epo nl, Fax: (+31-70) 340-3016	Authorized officer Mikael G:son Bergstrand

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INTERNATIONAL SEARCH REPORT

International application No.
PCT/GR 92/00015

C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
P,A	EP, A1, 0511712 (EASTMAN KODAK COMPANY), 4 November 1992 (04.11.92) -- --------	1-47

Form PCT/ISA/210 (continuation of second sheet) (July 1992)

SA 2772

INTERNATIONAL SEARCH REPORT
Information on patent family members

08/01/93

International application No.
PCT/GR 92/00015

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
EP-A1- 0511712	04/11/92	NONE	

Form PCT/ISA/210 (patent family annex) (July 1992)

(12) NACH DEM VERTRAG ÜBER DIE INTERNATIONALE ZUSAMMENARBEIT AUF DEM GEBIET DES PATENTWESENS (PCT) VERÖFFENTLICHTE INTERNATIONALE ANMELDUNG

(19) Weltorganisation für geistiges Eigentum
Internationales Büro

(43) Internationales Veröffentlichungsdatum
15. März 2007 (15.03.2007)

PCT

(10) Internationale Veröffentlichungsnummer
WO 2007/028833 A2

(51) Internationale Patentklassifikation: Nicht klassifiziert

(21) Internationales Aktenzeichen: PCT/EP2006/066223

(22) Internationales Anmeldedatum: 11. September 2006 (11.09.2006)

(25) Einreichungssprache: Deutsch

(26) Veröffentlichungssprache: Deutsch

(30) Angaben zur Priorität:
05019665.8 9. September 2005 (09.09.2005) EP

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WO 2007/028833 A2

(54) Title: METHOD FOR ACTIVATING A NUCLEIC ACID FOR A POLYMERASE REACTION

(54) Bezeichnung: VERFAHREN ZUR AKTIVIERUNG EINER NUKLEINSÄURE FÜR EINE POLYMERASE-REAKTION

(57) Abstract: The present invention relates to a method for activating a nucleic acid for a polymerase reaction, involving the following steps: (a) heating a nucleic acid to a temperature of 55 °C to 80 °C, (b) cooling the nucleic acid to a temperature at which a polymerase does not show any significant drop in activity and (c) starting the polymerase reaction by adding a heat-labile polymerase to the nucleic acid.

(57) Zusammenfassung: Die vorliegende Erfindung betrifft ein Verfahren zur Aktivierung einer Nukleinsäure für eine Polymerase-Reaktion mit den Schritten: (a) Erwärmen einer Nukleinsäure auf eine Temperatur von 55°C bis 80°C, (b) Abkühlen der Nukleinsäure auf eine Temperatur, bei der eine Polymerase keine wesentliche Abnahme der Aktivität zeigt, und (c) Starten der Polymerase-Reaktion durch Zugabe einer hitzelabilen Polymerase zu der Nukleinsäure.

Verfahren zur Aktivierung einer Nukleinsäure für eine Polymerase-Reaktion

Die vorliegende Erfindung betrifft ein Verfahren zur Aktivierung einer Nukleinsäure, insbesondere einer Desoxyribonukleinsäure (DNA), für eine Polymerase-Reaktion, insbesondere eine Strand-Displacement-Reaktion (Strangverdrängungsreaktion).

Unter einer Polymerase-Reaktion im Sinne der Erfindung wird die Polymerase-Aktivität einer Nukleinsäure-Polymerase verstanden, also das Polymerisieren von Nukleotiden an ein freies 3'-OH-Ende wobei der komplementäre Strang als Template dient. Dabei kann ein eventuell 3' von dem freien 3'-OH-Ende liegender Strang entweder verdrängt werden kann (Strand-Displacement-Reaktion, siehe unten) oder dieser durch eine 5'-3'-Exonuklease-Aktivität der Polymerase auch abgebaut und durch den neu synthetisierten Strang ersetzt werden kann (beispielsweise in einer Nick-Displacement-Reaktion).

Eine Strand-Displacement-Reaktion (SDR) ist eine Methode, bei der eine Polymerase-Reaktion mit Oligonukleotiden gestartet wird, wobei während der Reaktion ein Abschälen eines „alten“ Stranges („Strand-Displacement“) einer doppelsträngigen Nukleinsäure von dem anderen „alten“ (komplementären) Strang bewirkt wird, um ein Binden von Oligonukleotiden an den anderen „alten“ Strang zu ermöglichen. Wichtig für die Reaktion ist die Initiierung, wobei man verschiedene Techniken unterscheiden kann:

(A) Eine Trennung der beiden hybridisierenden Nukleinsäure-Stränge (z.B. DNA-Stränge) kann durch eine Hitzedenaturierung bei 95°C erfolgen. Bei dieser Temperatur werden nachweislich die beiden DNA-Stränge voneinander getrennt, so dass Oligonukleotide an den denaturierten (d.h. voneinander getrennten) DNA-Strängen binden können. Die Initiation der SDR kann dann erfolgen (z.B. Protokoll des GenomiPhi-Kits, Amersham Biosciences GmbH, Freiburg i. Br., Deutschland). Dieses Verfahren hat jedoch einen wesentlichen Nachteil: Eine Erhitzung auf 95°C führt zu einer Schädigung der DNA, z.B. durch Depurinisierung oder Strangbrüche (Suzuki T., Ohsumi S., Makino, K. (1994), *Mechanistic studies on depurination and apurinic site chain breakage in oligodeoxyribonucleotides*, Nucleic Acid Res. 22(23): 4997-5003).

(B) Eine Trennung der beiden hybridisierenden Nukleinsäure-Stränge (DNA-Stränge) kann auch durch eine Alkali-Denaturierung (Protokoll des REPLI-g Kits, QIAGEN GmbH, Hilden, Deutschland) erfolgen. Dies hat jedoch den Nachteil, dass nach der Alkali-Zugabe neutralisiert werden muss. Dies bedeutet zusätzliche Pipettierschritte und eine Veränderung im Reaktionsmilieu.

(C) Eine nächste Methode versucht keine Strangtrennung zu erreichen, sondern verwendet Endonukleasen, um Einzelstrangbrüche einzufügen, an deren 3´-OH Ende die Polymerase-Reaktion starten kann (vergl. z.B. US 6,884,586).

(D) Eine vierte Methode schließlich verwendet keine Denaturierung, wie z.B. von Notomi T., Okayama H., Masubuchi H., Yonekawa T., Watanabe K., Amino N., Hase T. (2000), *Loop-mediated isothermal amplification of DNA*, Nucleic Acids Res. 15; 28(12):E63 beschrieben. Dies führt jedoch zu deutlich schlechteren Ergebnissen, da vermutlich die in der DNA enthalten Einzelstrangbrüche für die Verlängerung in einer SDR verwendet werden.

Aufgabe der vorliegenden Erfindung ist es daher, ein Verfahren zur Aktivierung einer Nukleinsäure, insbesondere einer Desoxyribonukleinsäure (DNA), für eine Strand-Displacement-Reaktion (Strangverdrängungsreaktion) anzugeben, das die oben beschriebenen Nachteile des Standes der Technik nicht aufweist. Diese Aufgabe löst die Erfindung durch ein Verfahren zur Aktivierung einer Nukleinsäure für eine Polymerase-Reaktion mit den Schritten:

(a) Erwärmen einer Nukleinsäure auf eine Temperatur von 55°C bis 80°C,

(b) Abkühlen der Nukleinsäure auf eine Temperatur, bei der eine Polymerase keine wesentliche Abnahme der Aktivität zeigt, und

(c) Starten der Polymerase-Reaktion durch Zugabe einer Polymerase zu der Nukleinsäure.

Die Verwendung findende Polymerase kann hitzelabil oder hitzestabil sein. Bei Verwendung einer hitzestabilen Polymerase kann das erfindungsgemäße Verfahren zur Aktivierung einer Nukleinsäure für eine Polymerase-Reaktion alternativ den Schritt:

(a) Erwärmen der Nukleinsäure zusammen mit einer hitzestabilen Polymerase auf eine Temperatur von 55°C bis 80°C

aufweisen.

Weitere vorteilhafte Ausgestaltungen der vorliegenden Erfindung sind in den Ansprüchen, der Beschreibung, den Beispielen und der Zeichnung angegeben.

Nachfolgend werden einige der verwendeten Begriffe näher erläutert.

Strand-Displacement-Reaktion (SDR): Unter Strand-Displacement-Reaktion wird hier jede Reaktion verstanden, bei der eine Polymerase verwendet wird, die eine Strand-Displacement Aktivität aufweist, oder bei der eine Reaktionsbedingung verwendet wird, die ein Strand-Displacement ermöglicht. Hierzu zählen z.B. die Strand-Displacement-Amplification (SDA) genauso wie die Multiple-Displacement-Amplification (MDA) oder die Rolling-Circle-Amplification (RCA) sowie alle Unterformen dieser Reaktionen, wie z.B. Restriction-aided RCA (RCA-RCA) oder MDA mit *nested* Primern, lineare und exponentielle Strand-Displacement-Reaktionen oder auch Helicase-Dependent Amplification (vergl. z.B. die europäischen Patentanmeldungen Nr. 20050112639, 20050074804, 20050069939 und 20050069938, sowie Wang G., Maher E., Brennan C., Chin L., Leo C., Kaur M., Zhu P., Rook M., Wolfe J.L., Makrigiorgos G.M. (2004), *DNA amplification method tolerant to sample degradation*, Genome Res. Nov;14(11):2357-2366; Milla M.A., Spears P.A., Pearson R.E., Walker G.T. (1998), *Use of the restriction enzyme AvaI and exo-Bst polymerase in strand displacement amplification*, Biotechniques Mar;24(3):392-396; Nagamine K., Watanabe K., Ohtsuka K., Hase T., Notomi T. (2001), *Loop-mediated isothermal amplification reaction using a nondenatured template*, Clin Chem. 47(9):1742-1743; Notomi et al 2001 (siehe oben); Lage J.M., Leamon J.H., Pejovic T., Hamann S., Lacey M., Dillon D., Segraves R., Vossbrinck B., Gonzalez A., Pinkel D., Albertson D.G., Costa J., Lizardi P.M. (2003), *Whole genome analysis of genetic alterations in small DNA samples using hyperbranched strand displacement amplification and array-CGH*, Genome Res.13(2):294-

307; und Vincent M., Xu Y., Kong H. (2004), *Helicase-dependent isothermal DNA amplification*, EMBO Rep. 5(8):795-800).

Strand-Displacement-Polymerase: Zu den Strand-Displacement-Polymerasen gehören alle Polymerasen, die ein Strand-Displacement durchführen können. Hierzu zählen Enzyme, wie z.B. phi29-DNA-Polymerase, Cp-1-DNA-Polymerase, PRD1-DNA-Polymerase, phi15-DNA-Polymerase, phi21-DNA-Polymerase, PZE-DNA-Polymerase, PZA-DNA-Polymerase, Nf-DNA-Polymerase, M2Y-DNA-Polymerase, B103-DNA-Polymerase, SF5-DNA-Polymerase, GA-1-DNA-Polymerase, Cp-5-DNA-Polymerase, Cp-7-DNA-Polymerase, PR4-DNA-Polymerase, PR5-DNA-Polymerase, PR722-DNA-Polymerase, L17-DNA-Polymerase, Klenow DNA-Polymerase, Vent DNA Polymerase, Deep Vent DNA Polymerase, Bst DNA Polymerase, 9oNm™ DNA Polymerase, Polymerase III-Systeme und Bca DNA Polymerase. Die Strand-Dipslacement-Polymerasen können auch in mutierter Form vorliegen, z.B. als sogenannte exominus-Varianten (d.h. ohne Exonuklease Aktivität).

DNA: Desoxyribonukleinsäure (DNA) kommt natürlicherweise in Organismen vor, kann aber auch außerhalb von Organismen vorkommen oder diesem hinzugefügt worden sein. Die Länge der DNA kann unterschiedlich sein. Die DNA kann durch Veränderungen modifiziert sein. Die Basen der DNA können modifiziert sein. Die DNA kann Basenanaloga (z.B. auch non-Purin oder non-Pyrimidin Analoga) oder Nukleotidanaloga (z.B. PNA) enthalten. Die DNA kann Anhänge enthalten, wie z.B. Proteine oder Aminosäuren.

Die vorliegende Erfindung betrifft somit ein Verfahren zur Aktivierung einer Nukleinsäure (insbesondere einer doppelsträngigen DNA) für eine Strand-Displacement-Reaktion, wobei das Verfahren die folgenden Schritte umfasst: (a) Erwärmen der Nukleinsäure auf eine (im Vergleich zu herkömmlichen Verfahren moderate) Temperatur von 55°C bis 80°C; (b) Abkühlen der Nukleinsäure auf eine Temperatur, bei der eine Polymerase keine wesentliche Abnahme der Aktivität zeigt; und (c) Starten der Strand-Displacement-Reaktion durch Zugabe einer Polymerase zu der Nukleinsäure. Die bei einem bisher verwendeten Verfahren eingesetzte hohe Temperatur von 95°C, die sich, wie oben beschrieben, insofern negativ auswirkt, als eine eingesetzte Nukleinsäure (z.B. DNA) erheblich durch Strangbrüche und Depurinisierung) geschädigt wird, kann durch das erfindungsgemäße Verfahren vermieden werden. Auch auf die bisher häufig angewandte Trennung doppelsträngiger Nukleinsäuren mittels Alkalibehandlung mit den entsprechenden nachteiligen Begleitumständen kann durch

das neue Verfahren verzichtet werden. Das erfindungsgemäße Verfahren bietet somit eine die eingesetzte Nukleinsäure schonende Möglichkeit, eine Strand-Displacement-Reaktion vorzubereiten und durchzuführen. Das erfindungsgemäße Verfahren wird bevorzugt dann eingesetzt, wenn eine hitzelabile Polymerase zum Einsatz kommt. Im Falle der vorliegenden Erfindung wird eine Polymerase dann als hitzelabil bezeichnet, wenn diese nach einer Behandlung von 10 Minuten bei einer Temperatur von 65°C nur noch eine Aktivität von maximal 20 % der Ausgangsaktivität aufweist, d.h. wenn die Polymerase zu mindestens 80 % inaktiviert worden ist.

Die oben genannte Variante wird bevorzugt dann eingesetzt, wenn eine hitzelabile Polymerase zum Einsatz kommt. Wird dagegen eine hitzestabile Polymerase verwendet, die eine zumindest kurzfristige Erwärmung auf bis zu 80°C, bevorzugt auf bis zu 70°C und besonders bevorzugt auf bis zu 65°C ohne nennenswerte Einbuße ihrer Aktivität übersteht, kann diese bereits dem Schritt (a) zugesetzt werden, und eine Abkühlung des Reaktionsansatzes vor der Polymerasezugabe kann unterbleiben. Das alternative Verfahren umfasst daher den Schritt (a) Erwärmen der Nukleinsäure zusammen mit einer hitzestabilen Polymerase auf eine (im Vergleich zu herkömmlichen Verfahren moderate) Temperatur von 55°C bis 80°C. Unter hitzestabilen Polymerasen im Sinne der vorliegenden Erfindung werden alle nicht-hitzelabilen Polymerasen verstanden.

Die moderate Temperatur, bei der Zellen oder isolierte DNA erhitzt werden, liegt zwischen 55°C und 80°C, bevorzugt bei 60°C und 70°C und besonders bevorzugt bei 65°C. Die Erhitzung der DNA oder der Zellen kann beispielsweise direkt im SDR-Reaktionsgemisch erfolgen.

Die Erfindung beschreibt somit eine Aktivierung einer Nukleinsäure (insbesondere DNA) für eine Strand-Displacement Reaktion durch einen moderaten Erhitzungsschritt. Die erfindungsgemäße Methode umfasst bei Verwendung von isolierter Nukleinsäure (DNA) und einer hitzelabilen Strand-Displacement-Polymerase dementsprechend folgende Teilschritte: (1) Die Nukleinsäure (DNA) wird auf eine moderat hohe Temperatur erhitzt. (2) Die Nukleinsäure (DNA) wird abgekühlt, wobei die Temperatur nach dem Abkühlvorgang maximal eine Höhe haben darf, bei der die Polymerase noch nicht deutlich an Aktivität verliert. Die Nukleinsäure wird bevorzugt auf eine Temperatur von 4°C bis 45°C abgekühlt, besonders bevorzugt auf einen Bereich von 15°C bis 42°C und ganz besonders bevorzugt auf

einen Bereich von 25°C und 37°C. (3) Die SDR-Reaktion wird durch Zugabe der (hitzelabilen) Polymerase gestartet.

Die oben vorgestellte Variante wird bevorzugt dann eingesetzt, wenn eine hitzelabile Polymerase verwendet wird. Wird dagegen eine hitzestabile Polymerase eingesetzt, kann diese bereits dem Schritt (1) zugegeben werden.

Das erfindungsgemäße Verfahren kann aber nicht nur für reine bzw. gereinigte DNA verwendet werden, sondern auch für DNA, die noch in einem Zellverbund enthalten ist. Die Methode für die Verwendung von DNA, die noch im Zellverbund enthalten ist, und einer hitzelabilen Strand-Displacement-Polymerase hat dementsprechend folgende Teilschritte: (1) Die DNA-haltigen Zellen werden auf eine moderat hohe Temperatur erhitzt. (2) Die DNA-haltigen Zellen werden abgekühlt, wobei die Temperatur nach dem Abkühlvorgang maximal eine Höhe haben darf, bei der die Polymerase noch nicht deutlich an Aktivität verliert. (3) Die SDR-Reaktion wird durch Zugabe der Polymerase gestartet.

Bei beiden Methoden kann auch die hitzelabile Strand-Displacement-Polymerase auch durch eine hitzestabile Strand-Displacement-Polymerase ersetzt werden. Dann kann der Teilschritt (1) jeweils direkt mit der Polymerase durchgeführt werden.

In der Zeichnung zeigen

Fig. 1 die Ausbeute der Reaktionen aus Beispiel 1;
Fig. 2 die Ct-Werte der Real-time PCR aus Beispiel 1;
Fig. 3 die Ausbeute der Reaktionen aus Beispiel 2;
Fig. 4 die Ct-Werte der Real-time PCR aus Beispiel 2;
Fig. 5 die Ct-Werte der Real-time PCR aus Beispiel 3;
Fig. 6 die Ausbeute der Reaktionen aus Beispiel 4; und
Fig. 7 die Ct-Werte der Real-time PCR aus Beispiel 4.
Fig. 8 die Ct-Werte der Real-Time PCR aus Beispiel 5.
Fig. 9 die Ct-Werte der Real-Time PCR des Locus 11/12 aus Beispiel 6.
Fig. 10 die Ct-Werte der Real-Time PCR des Locus 665 aus Beispiel 6.

Die Erfindung wird nachfolgend anhand von Beispielen näher erläutert.

Beispiel 1

Das Beispiel soll zeigen, dass durch einen einfachen Temperaturaktivierungsschritt eine SDR (hier eine Multiple-Displacement-Amplification, MDA) ausgehend von Vollblut ermöglicht wird, die hinsichtlich der DNA-Ausbeute und DNA-Qualität vergleichbar zu einer Reaktion nach dem Stand der Technik (Kontrollreaktion) ist.

Erfindungsgemäße Reaktion: Die MDA-Reaktion wurde mit den REPLI-g Reagenzien (QIAGEN GmbH, Hilden, Deutschland) durchgeführt. Jeweils 0,5, 1 und 2 µl Vollblut, stabilisiert durch EDTA oder Citrat, wurden mit 12,5 µl 4x Reaction Mix (dieser enthält Oligonukleotide mit einer Zufallssequenz, Reaktionspuffer und dNTPs) und Wasser auf ein Volumen von 39,5 µl aufgefüllt und anschließend auf 65°C erwärmt. Nach Abkühlen auf Raumtemperatur (ca. 20-25°C) wurde die DNA-Polymerase aus dem REPLI-g Kit zugegeben. Die Reaktion wurde dann für 6 h bei 30°C durchgeführt.

Kontroll-Reaktion nach dem Stand der Technik: Die MDA-Reaktion wurde mit den REPLI-g Reagenzien (QIAGEN) durchgeführt: 0,5 µl Vollblut, stabilisiert durch EDTA, wurden mit 2,5 µl phosphatgepufferter Salzlösung (PBS, aus dem REPLI-g Kit von QIAGEN) versetzt. Anschließend wurden 3,5 µl des frisch angesetzten Denaturierungspuffers (360 mM KOH, 9 mM EDTA, 100 mM DTT) hinzu gegeben und für 10 min auf Eis inkubiert. Das Gemisch wurde nach der Inkubation mit Solution B (REPLI-g Kit) neutralisiert. Das Gemisch wurde mit 12,5 µl 4x Reaction Mix aus dem REPLI-g Kit (dieser enthält Oligonukleotide mit einer Zufallssequenz, Reaktionspuffer und dNTPs) und dest. Wasser auf ein Volumen von 49,5 µl aufgefüllt. Daraufhin wurde 0,5 µl der DNA-Polymerase aus dem REPLI-g Kit zugegeben. Die Reaktion wurde dann für 6 h bei 30°C durchgeführt.

Nach Ablauf der Reaktion wurde die DNA-Konzentration mit PicoGreen nach Hersteller-Protokoll (Molecular Probes Inc., Eugene, Oregon, USA) gemessen. 10 ng der MDA-DNA wurde für eine Real-time PCR (Polymerasekettenreaktion) eingesetzt. 4 verschiedene Loci wurden auf ihre Repräsentanz im Amplifikat hin untersucht:

(a) eine Sequenz, die als Sat bezeichnet wurde
(Primer-Sequenzen:
Sat1.1 TCTTTCCACTCCATTGCAT und
Sat1.2 GGAATGGAATCAACCCAA

(b) eine Sequenz aus dem ß-Aktin Gen
Primer 1 GTCTCAAGTCAGTGTACAGG
Primer 2 GTGATAGCATTGCTTTCGTG

(c) eine Sequenz, die aus dem als „1004“ bezeichneten Locus entstammt
(Probe: TGATGGCATTACTGGCACTTTGAGTTTTAC,
Primer 1: GTCTTTAGCTGCTGAGGAAATG,
Primer 2: AGCAGAATTCTGCACATGACG) und

(d) eine Sequenz, die aus dem als „699“ bezeichneten Locus entstammt
(Probe: TGAACTGCTCCTTGGCAGGGATTT,
Primer 1: TGCTCCCTGTCCCATCTG,
Primer 2: AGACAGTATGCCTTTATTTCACCC).

Die Real-time PCR Reaktionen wurden im QuantiTect Master Mix (QIAGEN) entsprechend den Protokoll-Anweisungen durchgeführt.

Die Repräsentanz der Loci in der amplifizierten DNA wurde in Ct-Werten (Threshold-Cycles = Ct) gemessen. Der Ct-Wert ist der PCR-Zyklus in der Real-time PCR, an dem das Fluoreszenz-Signal zum ersten Mal messbar wird. Durch den Ct-Wert kann somit die relative Häufigkeit einer Sequenz in der Probe ermittelt werden. Ist beispielsweise ein Ct-Wert um 1 Zyklus kleiner als in einer Vergleichsprobe, so entspricht dieser Wert einer ca. 2-fach höheren DNA-Ausgangsmenge im Testansatz gegenüber dem Kontrollansatz für die gemessene Sequenz.

Das Ergebnis von Beispiel 1 lässt sich wie folgt zusammenfassen: (1) Die Ausbeute aus den Reaktionen, bei denen die DNA erfindungsgemäß durch einen 65°C-Schritt aktiviert wurde, ist vergleichbar zur Ausbeute der Kontroll-Reaktion. (2) Auch die Repräsentanz der Sequenzen in der amplifizierten DNA ist vergleichbar, wenn 0,5 µl Blut eingesetzt wird.

Lediglich die Sequenz des Sat-Locus ist niedriger als in der Kontroll-Reaktion. (3) Größere Volumina als 0,5 µl wirken hemmend auf die MDA Reaktion mit 65°C Aktivierung. Dies ist erkennbar an den höheren Ct-Werten (d.h. schlechtere Repräsentanz der Sequenzen in der amplifizierten DNA).

Die Ausbeute der Reaktionen aus Beispiel 1 ist in Fig. 1 graphisch dargestellt. Fig. 2 zeigt die Ct-Werte der Real-time PCR aus Beispiel 1.

Beispiel 2

Dieses Beispiel dient dazu zu zeigen, dass zu geringe Temperaturen bei einem Temperaturaktivierungsschritt die Qualität der DNA, die während der SDR entsteht, beeinträchtigen kann.

Erfindungsgemäße Reaktion: Die MDA-Reaktion wurde mit den REPLI-g Reagenzien (QIAGEN) durchgeführt: 0,5 µl Vollblut, stabilisiert durch EDTA, wurde mit 12,5 µl 4x Reaction Mix (dieser enthält Oligonukleotide mit einer Zufallssequenz, Reaktionspuffer und dNTPs) und Wasser auf ein Volumen von 39,5 µl aufgefüllt und durch verschiedene Temperaturen aktiviert (30, 40, 45, 50, 55, 60 bzw. 65°C). Nach Abkühlen auf Raumtemperatur (ca. 20-25°C) wurde die DNA-Polymerase aus dem REPLI-g Kit zugegeben. Die Reaktion wurde für 6 h bei 30°C durchgeführt.

Kontroll-Reaktion nach dem Stand der Technik: Die MDA-Reaktion wurde mit den REPLI-g Reagenzien (QIAGEN) durchgeführt: 0,5 µl Vollblut, stabilisiert durch EDTA, wurden mit 2,5 µl PBS (REPLI-g Kit) versetzt. Anschließend wurden 3,5 µl des frisch angesetzten Denaturierungspuffer (360 mM KOH, 9 mM EDTA, 100 mM DTT) hinzu gegeben und für 10 min auf Eis inkubiert. Das Gemisch wurde nach der Inkubation mit Solution B (REPLI-g Kit) neutralisiert. Das Gemisch wurde dann mit 12,5 µl 4x Reaction Mix aus dem REPLI-g Kit (dieser enthält Oligonukleotide mit einer Zufallssequenz, Reaktionspuffer und dNTPs) und dest. Wasser auf ein Volumen von 49,5 µl aufgefüllt. Daraufhin wurde 0,5 µl der DNA-Polymerase aus dem REPLI-g Kit zugegeben. Die Reaktion wurde für 6 h bei 30°C durchgeführt.

Nach Ablauf der Reaktion wurde die DNA-Konzentration mit PicoGreen nach Hersteller-Protokoll (Molecular Probes) gemessen. 10 ng der MDA-DNA wurden für eine Real-time PCR eingesetzt. 2 verschiedene Loci wurden auf ihre Repräsentanz im Amplifikat hin untersucht:

(a) eine Sequenz, die aus dem als „1004“ bezeichneten Locus entstammt
(Probe: TGATGGCATTACTGGCACTTTGAGTTTTAC,
Primer 1: GTCTTTAGCTGCTGAGGAAATG und
Primer 2: AGCAGAATTCTGCACATGACG) sowie

(b) eine Sequenz, die aus dem als „699“ bezeichneten Locus entstammt
(Probe: TGAACTGCTCCTTGGCAGGGATTT,
Primer 1: TGCTCCCTGTCCCATCTG,
Primer 2: AGACAGTATGCCTTTATTTCACCC).

Die Real-time PCR Reaktionen wurden im QuantiTect Master Mix (QIAGEN) entsprechend den Protokoll-Anweisungen durchgeführt.

Die Repräsentanz der Loci in der amplifizierten DNA wurde in Ct-Werten (Threshold-Cycles = Ct) gemessen. Der Ct-Wert ist der PCR-Zyklus in der Real-time PCR, an dem das Fluoreszenz-Signal zum ersten Mal messbar wird. Durch den Ct-Wert kann somit die relative Häufigkeit einer Sequenz in der Probe ermittelt werden. Ist beispielsweise ein Ct-Wert um 1 Zyklus kleiner als in einer Vergleichsprobe, so entspricht dieser Wert einer ca. 2-fach höheren DNA-Ausgangsmenge im Testansatz gegenüber dem Kontrollansatz für die gemessene Sequenz.

Das Ergebnis von Beispiel 2 lässt sich wie folgt zusammenfassen: (1) Die Ausbeute aus den Reaktionen, bei denen die DNA durch einen 30 bis 65°C-Schritt aktiviert wurde, ist vergleichbar zur der Kontroll-Reaktion. (2) Auch die Repräsentanz der Sequenzen in der amplifizierten DNA ist nur etwas schlechter als bei der Kontrollreaktion, wenn die Aktivierung bei 60 oder 65°C durchgeführt wurde. Bei einer Aktivierung der DNA für die Strand-Displacement Reaktion unter 60°C wird die Repräsentanz der betrachteten Sequenzen schlechter.

Die Ausbeute der Reaktionen aus Beispiel 2 ist in Fig. 3 graphisch dargestellt. Fig. 4 zeigt die Ct-Werte der Real-Time PCR aus Beispiel 2.

Beispiel 3

Mit diesem Beispiel soll gezeigt werden, dass innerhalb bestimmter Temperaturgrenzen eine einfache Temperaturaktivierung vor der SDR die Qualität der in der SDR entstehenden DNA im Vergleich zu einer Reaktion nach dem Stand der Technik (Kontrollreaktion) nicht beeinträchtigt.

Erfindungsgemäße Reaktion: Die MDA-Reaktion wurde mit den REPLI-g Reagenzien (QIAGEN) durchgeführt. 0,5 µl Vollblut, stabilisiert durch EDTA, wurden mit 12,5 µl 4x Reaction Mix (dieser enthält Oligonukleotide mit einer Zufallssequenz, Reaktionspuffer und dNTPs) und Wasser auf ein Volumen von 39,5 µl aufgefüllt und durch verschiedene Temperaturen aktiviert (60, 65 und 70°C). Nach Abkühlen des Reaktionsansatzes auf Raumtemperatur (ca. 20-25 °C) wurde die DNA-Polymerase aus dem REPLI-g Kit zugegeben. Die Reaktion wurde für 6 h bei 30°C durchgeführt.

Kontroll-Reaktion nach dem Stand der Technik: Die MDA-Reaktion wurde mit den REPLI-g Reagenzien (QIAGEN) durchgeführt: 0,5 µl Vollblut, stabilisiert durch EDTA, wurden mit 2,5 µl PBS (REPLI-g Kit) versetzt. Anschließend wurden 3,5 µl des frisch angesetzten Denaturierungspuffers (360 mM KOH, 9 mM EDTA, 100 mM DTT) hinzu gegeben und für 10 min auf Eis inkubiert. Das Gemisch wurde nach der Inkubation mit Solution B (REPLI-g Kit) neutralisiert. Das Gemisch wurde mit 12,5 µl 4x Reaction Mix aus dem REPLI-g Kit (dieser enthält Oligonukleotide mit einer Zufallssequenz, Reaktionspuffer und dNTPs) und mit dest. Wasser auf ein Volumen von 49,5 µl aufgefüllt. Daraufhin wurden 0,5 µl der DNA-Polymerase aus dem REPLI-g Kit zugegeben. Die Reaktion wurde für 6 h bei 30°C durchgeführt.

Nach Ablauf der Reaktion wurde die DNA-Konzentration mit PicoGreen nach Hersteller-Protokoll (Molecular Probes) gemessen. 10 ng der MDA-DNA wurden für eine Real-time PCR eingesetzt. 4 verschiedene Loci wurden auf ihre Repräsentanz im Amplifikat hin untersucht:

(a) eine Sequenz, die aus dem als „1004“ bezeichneten Locus entstammt
(Probe: TGATGGCATTACTGGCACTTTGAGTTTTAC,
Primer 1: GTCTTTAGCTGCTGAGGAAATG,
Primer 2: AGCAGAATTCTGCACATGACG) und

(b) eine Sequenz, die aus dem als „699“ bezeichneten Locus entstammt
(Probe: TGAACTGCTCCTTGGCAGGGATTT,
Primer 1: TGCTCCCTGTCCCATCTG,
Primer 2: AGACAGTATGCCTTTATTTCACCC).

c) eine Sequenz, die als Sat bezeichnet wurde
(Primer-Sequenzen:
Sat1.1 TCTTTCCACTCCATTGCAT und
Sat1.2 GGAATGGAATCAACCCAA

(d) eine Sequenz aus dem ß-Aktin Gen
Primer 1 GTCTCAAGTCAGTGTACAGG
Primer 2 GTGATAGCATTGCTTTCGTG

Die Real-time PCR Reaktionen wurden im QuantiTect Master Mix (QIAGEN) entsprechend den Protokoll-Anweisungen durchgeführt.

Die Repräsentanz der Loci in der amplifizierten DNA wurde in Ct-Werten (Threshold-Cycles = Ct) gemessen. Der Ct-Wert ist der PCR-Zyklus in der Real-time PCR, an dem das Fluoreszenz-Signal zum ersten Mal meßbar wird. Durch den Ct-Wert kann somit die relative Häufigkeit einer Sequenz in der Probe ermittelt werden. Ist beispielsweise ein Ct-Wert um 1 Zyklus kleiner als in einer Vergleichsprobe, so entspricht dieser Wert einer ca. 2-fach höheren DNA-Ausgangsmenge im Testansatz gegenüber dem Kontrollansatz für die gemessene Sequenz.

Das Ergebnis lässt sich wie folgt zusammenfassen. Die Repräsentanz der Sequenzen in der amplifizierten DNA ist bei dem erfindungsgemäßen Ansatz teilweise besser als bei der Kontrollreaktion, wenn die Aktivierung bei 60°C, 65°C oder 70°C durchgeführt wurde.

Fig. 5 zeigt die Ct-Werte der Real-Time PCR-Analyse von DNA aus der SDR-Reaktion von Beispiel 3.

Beispiel 4

Dieses Beispiel dient dazu zu zeigen, dass durch einen einfachen Temperaturaktivierungsschritt eine SDR (hier eine Multiple Displacement Amplification, MDA), ausgehend von isolierter genomischer DNA, ermöglicht wird, die hinsichtlich DNA-Ausbeute und DNA-Qualität vergleichbar zu einer Reaktion nach dem Stand der Technik (Kontrollreaktion) ist.

Erfindungsgemäße Reaktion: Die MDA-Reaktion wurde mit den REPLI-g Reagenzien (QIAGEN) durchgeführt. 2,5 µl einer Lösung von genomischer DNA aus humanen Zellen (Konzentration: 4 ng/µl) wurden mit 12,5 µl 4x Reaction Mix (dieser enthält Oligonukleotide mit einer Zufallssequenz, Reaktionspuffer und dNTPs) und Wasser auf ein Volumen von 39,5 µl aufgefüllt und durch einen Inkubationsschritt bei 65°C aktiviert. Nach Abkühlen des Reaktionsansatzes auf Raumtemperatur (ca. 20-25 °C) wurde die DNA-Polymerase aus dem REPLI-g Kit zugegeben. Die Reaktion wurde für 6 h bei 30°C durchgeführt.

Kontroll-Reaktion nach dem Stand der Technik: Die MDA-Reaktion wurde mit den REPLI-g Reagenzien (QIAGEN) durchgeführt. 2,5 µl genomische DNA aus humanen Zellen (Konzentration: 4 ng/µl) wurden mit 2,5 µl frisch angesetztem Denaturierungspuffer (50 mM KOH, 1,25 mM EDTA) versetzt und für 3 min bei Raumtemperatur inkubiert. Das Gemisch wurde nach der Inkubation mit einer 1:10 Verdünnung der Solution B (REPLI-g Kit) neutralisiert. Das Gemisch wurde mit 12,5 µl 4x Reaction Mix aus dem REPLI-g Kit (dieser enthält Oligonukleotide mit einer Zufallssequenz, Reaktionspuffer und dNTPs) und dest. Wasser auf ein Volumen von 49,5 µl aufgefüllt. Daraufhin wurden 0,5 µl der DNA-Polymerase aus dem REPLI-g Kit zugegeben. Die Reaktion wurde für 6 h bei 30°C durchgeführt.

Nach Ablauf der Reaktion wurde die DNA-Konzentration mit PicoGreen nach Hersteller-Protokoll (Molecular Probes) gemessen. 10 ng der MDA-DNA wurde für eine Real-time PCR eingesetzt. 1 Locus wurde auf die Repräsentanz im Amplifikat hin untersucht:

(a) eine Sequenz, die aus dem als „1004“ bezeichneten Locus entstammt
(Probe: TGATGGCATTACTGGCACTTTGAGTTTTAC,
Primer 1: GTCTTTAGCTGCTGAGGAAATG,
Primer 2: AGCAGAATTCTGCACATGACG).

Die Real-time PCR Reaktionen wurden im QuantiTect Master Mix (QIAGEN) entsprechend den Protokoll-Anweisungen durchgeführt.

Die Repräsentanz der Loci in der amplifizierten DNA wurde in Ct-Werten (Threshold-Cycles = Ct) gemessen. Der Ct-Wert ist der PCR-Zyklus in der Real-time PCR, an dem das Fluoreszenz-Signal zum ersten Mal messbar wird. Durch den Ct-Wert kann somit die relative Häufigkeit einer Sequenz in der Probe ermittelt werden. Ist beispielsweise ein Ct-Wert um 1 Zyklus kleiner als in einer Vergleichsprobe, so entspricht dieser Wert einer ca. 2-fach höheren DNA-Ausgangsmenge im Testansatz gegenüber dem Kontrollansatz für die gemessene Sequenz.

Das Ergebnis war, dass die Ausbeute aus den Reaktionen, bei denen die DNA durch einen 65°C-Schritt aktiviert wurde, fast doppelt so hoch war wie bei der Kontrolle nach dem Stand der Technik. Die Repräsentanz der Sequenzen in der amplifizierten DNA ist in der erfindungsgemäßen Probe und der Kontrollprobe vergleichbar.

Die Ausbeute der Reaktionen aus Beispiel 4 ist in Fig. 6 graphisch dargestellt. Fig. 7 zeigt die Ct-Werte der Real-Time PCR aus Beispiel 4.

Beispiel 5

In diesem Beispiel wird gezeigt, dass durch einen einfachen Denaturierungsschritt bei zunehmend hohen Temperaturen bestimmte Sequenzen nicht mehr in einer SDR (hier eine Mutliple Displacement Amplification) amplifiziert werden können.

Test-Reaktionen: Die MDA-Reaktion wurde mit den REPLI-g Reagenzien (QIAGEN) durchgeführt: 10 ng isolierte DNA wurde bei Temperaturen von 75°C, 85°C oder 95°C für 10 min. inkubiert. Alternativ wurde in Kontrollreaktionen eine alkalische Denaturierung in einem KOH-Puffer durchgeführt. Nach der chemischen Denaturierung mit KOH wurde die Lösung neutralisiert, um die MDA Reaktionsbedingungen nicht zu beeinflussen. Anschließend an die Hitzebehandlung oder chemische Denaturierung wurden der DNA-haltigen Lösung 12,5 µl 4x Reaction Mix (dieser enthält Oligonukleotide mit einer Zufallssequenz, Reaktionspuffer und dNTPs) und Wasser auf ein Volumen von 39,5 µl aufgefüllt. Anschließend wurde den Reaktionsgemischen die DNA-Polymerase aus dem REPLI-g Kit zugegeben. Die Reaktion wurde für 6 h bei 30°C durchgeführt.

Nach Ablauf der Reaktion wurde die DNA-Konzentration mit PicoGreen nach Hersteller-Protokoll (Molecular Probes) gemessen. 10 ng der MDA-DNA wurde für eine Real-time PCR eingesetzt. 2 verschiedene Loci wurden auf ihre Repräsentanz im Amplifikat hin untersucht:

(a) eine Sequenz, die aus dem als 1004 bezeichneten Locus entstammt
(Probe: TGATGGCATTACTGGCACTTTGAGTTTTAC,
Primer 1: GTCTTTAGCTGCTGAGGAAATG,
Primer 2: AGCAGAATTCTGCACATGACG) und

(b) Sequenz, die aus dem als 699 bezeichneten Locus entstammt
(Probe:TGAACTGCTCCTTGGCAGGGATTT,
Primer 1: TGCTCCCTGTCCCATCTG,
Primer 2: AGACAGTATGCCTTTATTTCACCC).

Die Real-time PCR Reaktionen wurden im QuantiTect Master Mix (QIAGEN) entsprechend den Protokoll-Anweisungen durchgeführt.

Die Repräsentanz der Loci in der amplifizierten DNA wurde in Ct-Werten (Threshold-Cycles = Ct) gemessen. Der Ct-Wert ist der PCR-Zyklus in der Real-time PCR, an dem das Fluoreszenz-Signal zum ersten Mal messbar wird. Durch den Ct-Wert kann somit die relative Häufigkeit einer Sequenz in der Probe ermittelt werden. Ist beispielsweise ein Ct-Wert um 1 Zyklus kleiner als in einer Vergleichsprobe, so entspricht dieser Wert einer ca. 2fach höheren

DNA-Ausgangsmenge im Testansatz gegenüber dem Kontrollansatz für die gemessene Sequenz.

Ergebnis:

(1) Mit zunehmender Temperatur bei der Hitzebehandlung der DNA wurden immer höhere CT-Werte gemessen.
(2) Beide Loci verhalten sich unterschiedlcih auf die Hitzebehandlung: Im Falle des Locus 699 konnte eine Ct-Verschiebung von 9,6 Zyklen gemessen werden, vergleicht man die Werte bei einer 75°C bzw. 95°C Behandlung. Im Falle des Locus 1004 immerhin noch eine Ct-Verschiebung von 5 Zyklen gemessen werden. D.h. mit zunehmender Temperatur der Hitzebehandlung der DNA wurden im MDA Amplifikationsprodukt die gemessenen Loci immer schlechter nachgewiesen, obwohl in einer Einheitsmenge des Amplifikationsproduktes (10ng) für Real-time PCR eingesetzt wurde.

Eine Ct-Verschiebung von 9,6 bzw. 5 Zyklen entspricht einer 780fachen* bzw. 30fachen* geringeren Repräsentation der Sequenz 699 bzw. 1004 im Amplifikationsprodukt der DNA, die vor Amplifikation auf 95°C erhitzt wurde, verglichen zu einer bei 75°C behandelten DNA (* diese Werte legen zugrunde, dass in jedem Zyklus der real-time PCR eine Verdopplung stattfindet).

Fig. 8 zeigt die Ct-Werte der Real-Time PCR aus Beispiel 5.

Beispiel 6

Dieses Beispiel zeigt, bei welchen Temperaturen ein optimaler Temperaturaktivierungsschritt erfolgt, um eine möglichst gute Sequenz-Repräsentation bei einer SDR (hier eine Mutliple Displacement Amplification) zu erzielen.

Test-Reaktionen: Die MDA-Reaktion wurde mit den REPLI-g Reagenzien (QIAGEN) durchgeführt: 10 ng isolierte DNA wurde bei verschiedenen Temperaturen für 5 min inkubiert. Alternativ wurde in Kontrollreaktionen DNA in einem Mehrschrittverfahren

bestehend aus (1) Zugabe von KOH, (2) Inkubation der DNA in der KOH Lösung für 5 min und (3) Neutralisierung der alkalischen Lösung für die REPLI-g Reaktion bereitet.

Anschließend an die Hitzebehandlung oder chemische Denaturierung wurden der DNA-haltigen Lösung in einer REPLI-g Reaktion amplifiziert. Die Reaktion wurde für 8 h bei 33°C durchgeführt.

Nach Ablauf der Reaktion wurde die DNA-Konzentration mit PicoGreen nach Hersteller-Protokoll (Molecular Probes) gemessen. 10 ng der MDA-DNA wurde für eine Real-time PCR eingesetzt. 2 verschiedene Loci wurden auf ihre Repräsentanz im Amplifikat hin untersucht:
(a) eine Sequenz, die aus dem als 11/12 bezeichneten Locus entstammt
(Primer 1: TTTCTGTAACAGCTAAGGAC,
Primer 2: TAGGGTGCTTAGCTGTTAAC) und

(b) Sequenz, die aus dem als 665 bezeichneten Locus entstammt
(Primer 1: CTCTTGCTCAGCCTATATAC,
Primer 2: GTAGAAAATGTAGCCCATTAC.

Die Real-time PCR Reaktionen wurden im QuantiTect Master Mix (QIAGEN) entsprechend den Protokoll-Anweisungen durchgeführt.

Die Repräsentanz der Loci in der amplifizierten DNA wurde in Ct-Werten (Threshold-Cycles = Ct) gemessen. Der Ct-Wert ist der PCR-Zyklus in der Real-time PCR, an dem das Fluoreszenz-Signal zum ersten Mal messbar wird. Durch den Ct-Wert kann somit die relative Häufigkeit einer Sequenz in der Probe ermittelt werden. Ist beispielsweise ein Ct-Wert um 1 Zyklus kleiner als in einer Vergleichsprobe, so entspricht dieser Wert einer ca. 2fach höheren DNA-Ausgangsmenge im Testansatz gegenüber dem Kontrollansatz für die gemessene Sequenz.

Ergebnis:
(1) Mit zunehmender Temperatur bei der Hitzebehandlung der DNA wurden immer höhere CT-Werte gemessen.
(2) Die niedrigsten CT-Werte (also die beste Repräsentation der hier untersuchten Loci) ergibt sich bei einer thermischen Vorbehandlung bei einer Temperatur von 65°C bis 85°C.

(3) Bei diesen Loci wurden bei den Temperaturen 65-85°C bessere Ct-Werte gemessen als bei der Referenz-Behandlung mittels KOH.

(4) Durch den Vergleich Beispiel 5, bei dem bei 85°C bereits schlechtere CT-Werte gemessen wurden als bei der Referenzbehandlung mittels KOH, kann gefolgert werden, dass die optimale Behandlungstemperatur abhängig ist vom Locus im Genom einer Zelle.

Fig. 9 zeigt die Ct-Werte der Real-Time PCR des Locus 11/12 aus Beispiel 6. Fig. 10 zeigt die Ct-Werte der Real-Time PCR des Locus 665 aus Beispiel 6.

Patentansprüche

1. Verfahren zur Aktivierung einer Nukleinsäure für eine Polymerase-Reaktion mit den Schritten:

(a) Erwärmen einer Nukleinsäure auf eine Temperatur von 55°C bis 80°C,

(b) Abkühlen der Nukleinsäure auf eine Temperatur, bei der eine Polymerase keine wesentliche Abnahme der Aktivität zeigt, und

(c) Starten der Polymerase-Reaktion durch Zugabe einer Polymerase zu der Nukleinsäure.

2. Verfahren nach Anspruch 1, dadurch gekennzeichnet, dass die Polymerase eine hitzelabile Polymerase ist.

3. Verfahren nach Anspruch 1, dadurch gekennzeichnet, dass die Polymerase eine hitzestabile Polymerase ist.

4. Verfahren zur Aktivierung einer Nukleinsäure für eine Polymerase-Reaktion mit dem Schritt:

(a) Erwärmen der Nukleinsäure zusammen mit einer hitzestabilen Polymerase auf eine Temperatur von 55°C bis 80°C.

5. Verfahren nach Anspruch 1 oder 4, dadurch gekennzeichnet, dass die Polymerase-Reaktion eine Strand-Displacement-Reaktion ist.

6. Verfahren nach Anspruch 5, dadurch gekennzeichnet, dass die Strand-Displacement-Reaktion eine Multiple Displacement-Reaktion ist.

7. Verfahren nach einem der vorhergehenden Ansprüche, dadurch gekennzeichnet, dass die Nukleinsäure eine DNA ist.

8. Verfahren nach einem der vorhergehenden Ansprüche, dadurch gekennzeichnet, dass die Nukleinsäure in Schritt (a) auf eine Temperatur von 60°C bis 70°C erwärmt wird.

9. Verfahren nach Anspruch 8, dadurch gekennzeichnet, dass die Nukleinsäure in Schritt (a) auf eine Temperatur von 65°C erwärmt wird.

10. Verfahren nach Anspruch 1, dadurch gekennzeichnet, dass in Schritt (b) die Nukleinsäure auf eine Temperatur von 4°C bis 45°C abgekühlt wird.

11. Verfahren nach Anspruch 10, dadurch gekennzeichnet, dass in Schritt (b) die Nukleinsäure auf eine Temperatur von 15°C bis 42°C abgekühlt wird.

12. Verfahren nach Anspruch 11, dadurch gekennzeichnet, dass in Schritt (b) die Nukleinsäure auf eine Temperatur von 25°C bis 37°C abgekühlt wird.

13. Verfahren nach einem der vorhergehenden Ansprüche, dadurch gekennzeichnet, dass die Nukleinsäure in gereinigter Form in einer wässrigen Lösung vorliegt.

14. Verfahren nach einem der Ansprüche 1 bis 12, dadurch gekennzeichnet, dass die Nukleinsäure in einer Zelle vorliegt.

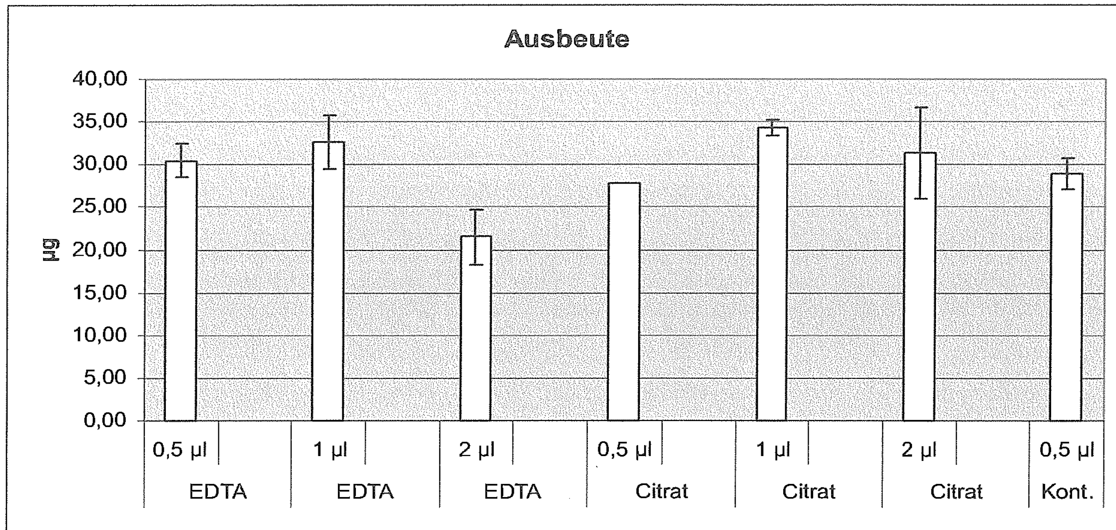

FIG. 1

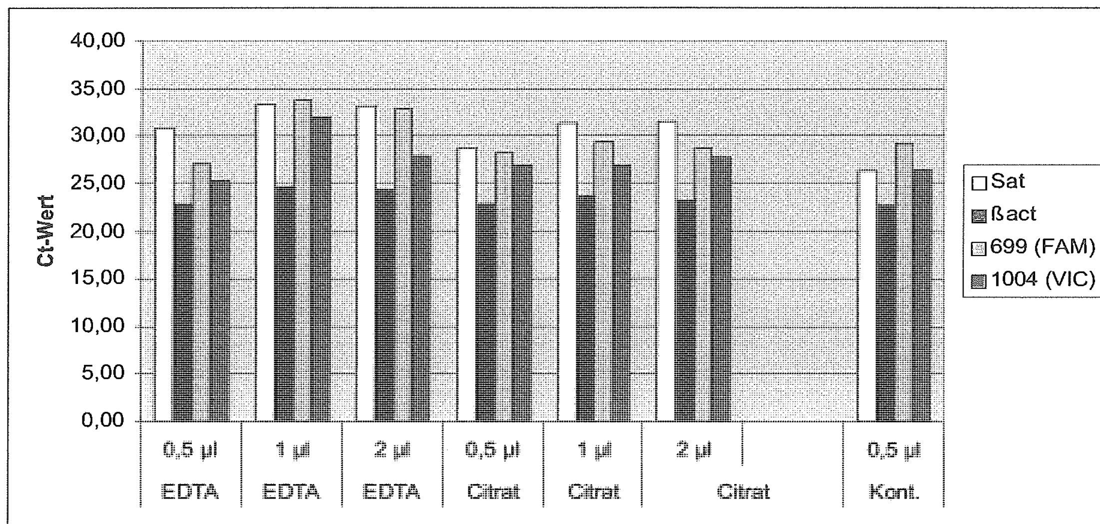

FIG. 2

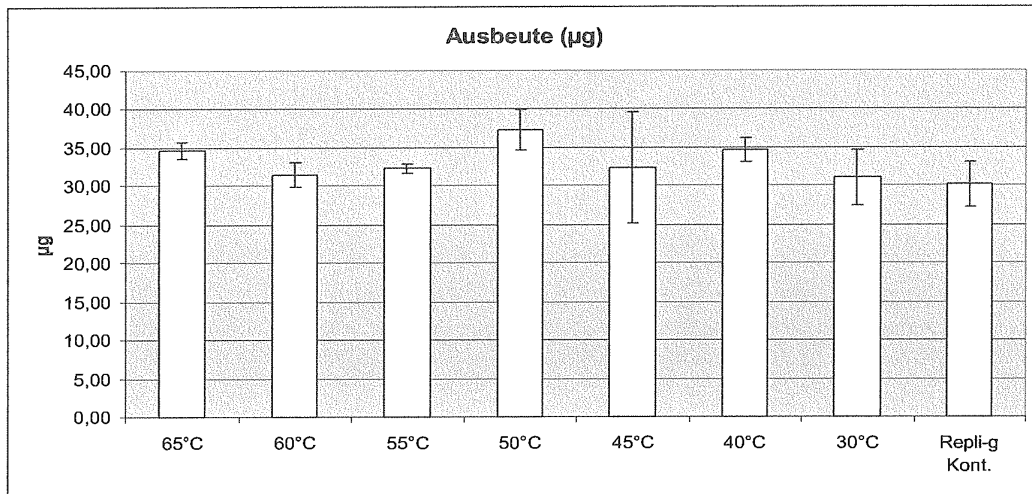

FIG. 3

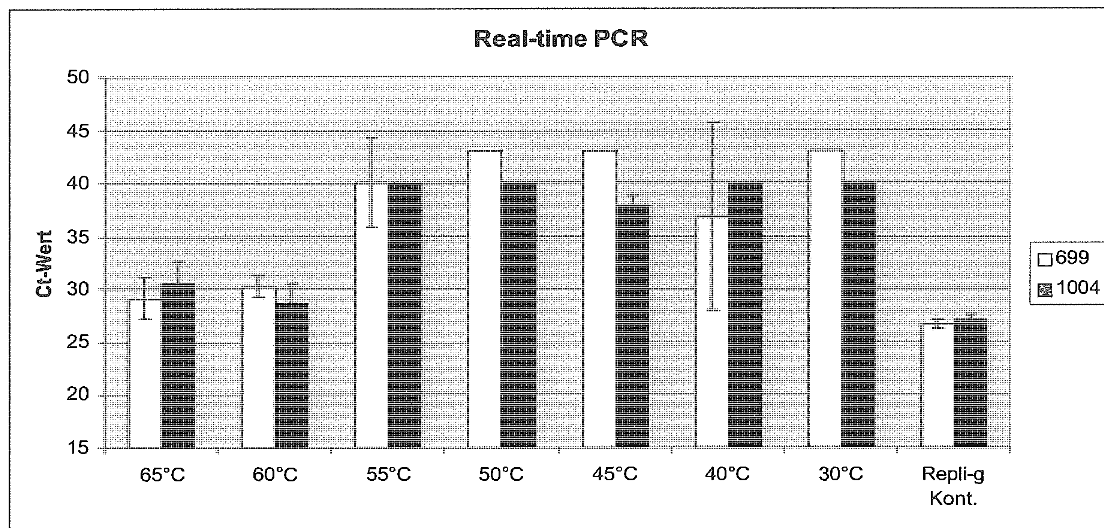

FIG. 4

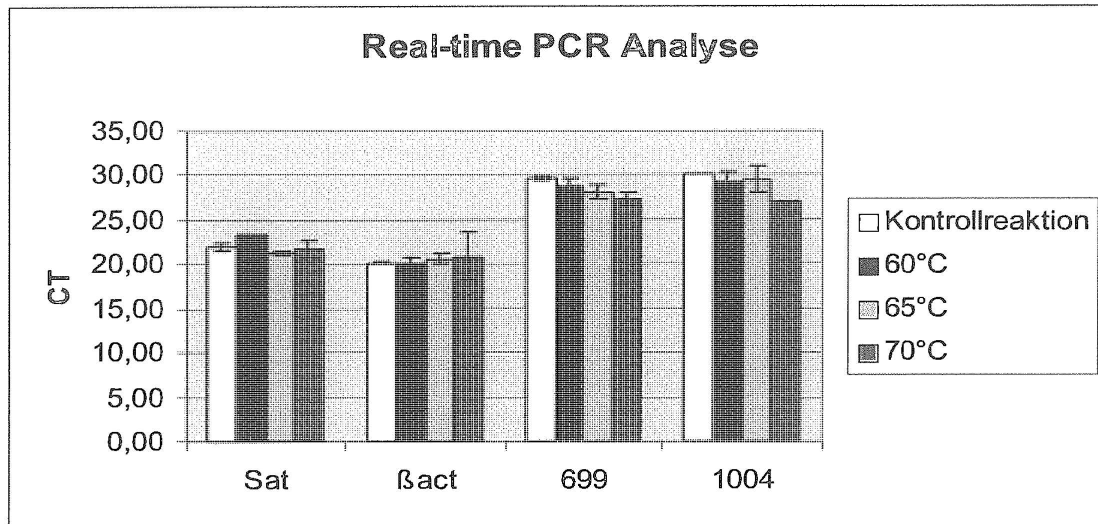

FIG. 5

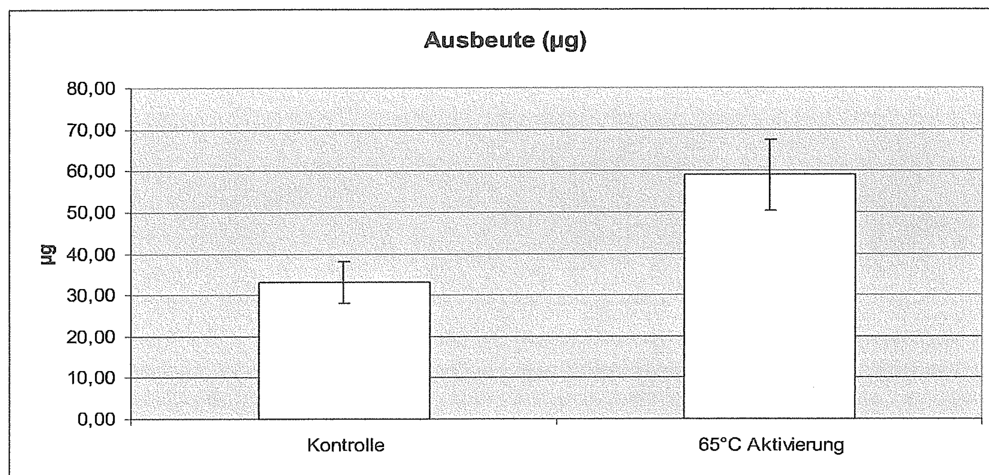

FIG. 6

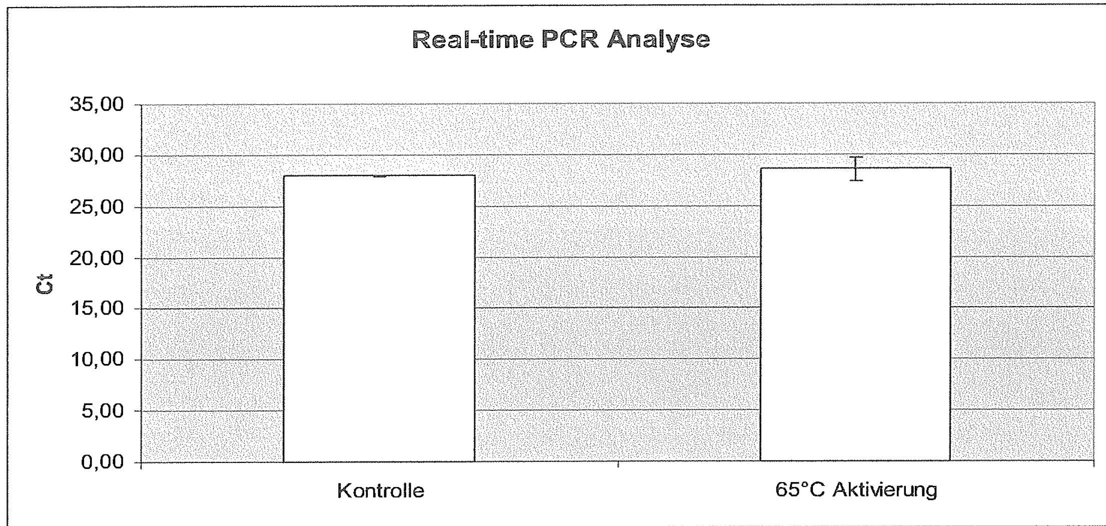

FIG. 7

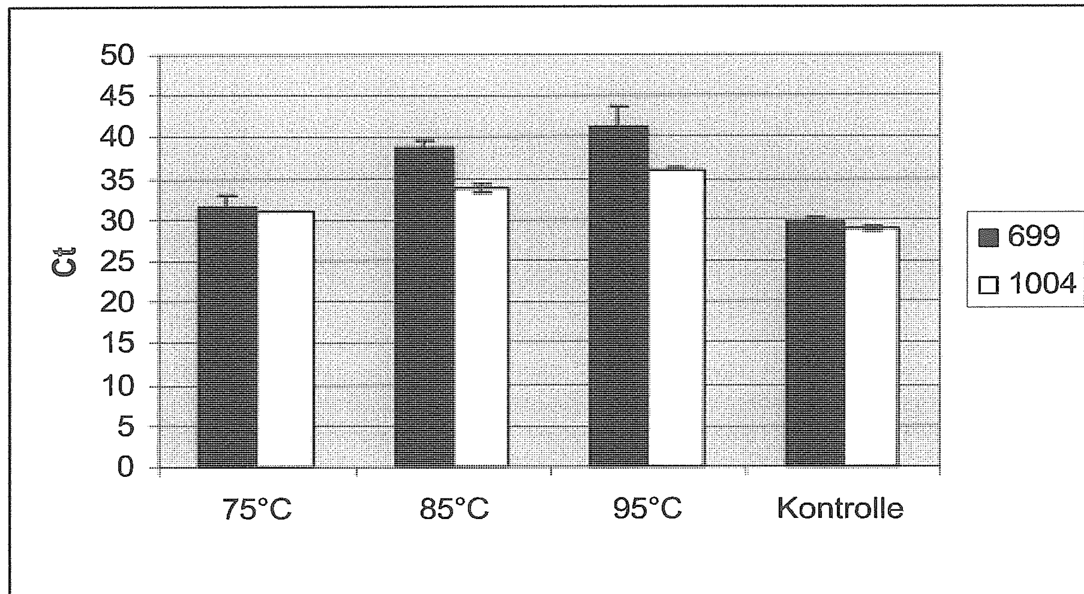

FIG. 8

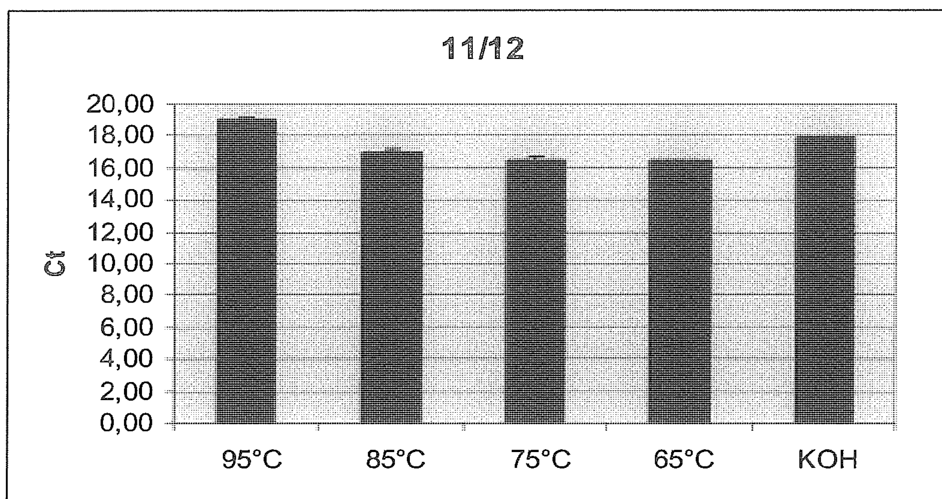

FIG. 9

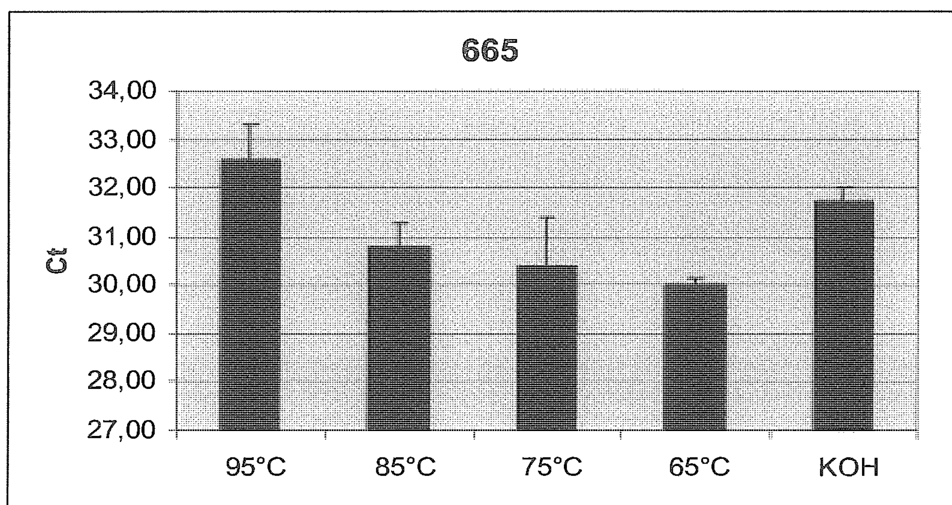

FIG. 10

(12) NACH DEM VERTRAG ÜBER DIE INTERNATIONALE ZUSAMMENARBEIT AUF DEM GEBIET DES PATENTWESENS (PCT) VERÖFFENTLICHTE INTERNATIONALE ANMELDUNG

(19) Weltorganisation für geistiges Eigentum
Internationales Büro

(43) Internationales Veröffentlichungsdatum
15. März 2007 (15.03.2007)

PCT

(10) Internationale Veröffentlichungsnummer
WO 2007/028833 A3

(51) Internationale Patentklassifikation:
C12Q 1/68 (2006.01)

(21) Internationales Aktenzeichen: PCT/EP2006/066223

(22) Internationales Anmeldedatum:
11. September 2006 (11.09.2006)

(25) Einreichungssprache: Deutsch

(26) Veröffentlichungssprache: Deutsch

(30) Angaben zur Priorität:
05019665.8 9. September 2005 (09.09.2005) EP

(71) Anmelder *(für alle Bestimmungsstaaten mit Ausnahme von US)*: **QIAGEN GMBH** [DE/DE]; Qiagen Str.1, 40724 Hilden (DE).

(72) Erfinder; und
(75) Erfinder/Anmelder *(nur für US)*: **KORFHAGE, Christian** [DE/DE]; Sepp-Herberger-Str. 6c, 40764 Langenfeld (DE). **LÖFFERT, Dirk** [DE/DE]; Bahlenstr. 8, 40589 Düsseldorf (DE).

(74) Gemeinsamer Vertreter: **QIAGEN GMBH**; IP & L, Post Code 870, Qiagen Str.1, 40724 Hilden (DE).

(81) Bestimmungsstaaten *(soweit nicht anders angegeben, für jede verfügbare nationale Schutzrechtsart)*: AE, AG, AL, AM, AT, AU, AZ, BA, BB, BG, BR, BW, BY, BZ, CA, CH, CN, CO, CR, CU, CZ, DE, DK, DM, DZ, EC, EE, EG, ES, FI, GB, GD, GE, GH, GM, HN, HR, HU, ID, IL, IN, IS, JP, KE, KG, KM, KN, KP, KR, KZ, LA, LC, LK, LR, LS, LT, LU, LV, LY, MA, MD, MG, MK, MN, MW, MX, MY, MZ, NA, NG, NI, NO, NZ, OM, PG, PH, PL, PT, RO, RS, RU, SC, SD, SE, SG, SK, SL, SM, SV, SY, TJ, TM, TN, TR, TT, TZ, UA, UG, US, UZ, VC, VN, ZA, ZM, ZW.

(84) Bestimmungsstaaten *(soweit nicht anders angegeben, für jede verfügbare regionale Schutzrechtsart)*: ARIPO (BW, GH, GM, KE, LS, MW, MZ, NA, SD, SL, SZ, TZ, UG, ZM, ZW), eurasisches (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), europäisches (AT, BE, BG, CH, CY, CZ, DE, DK, EE, ES, FI, FR, GB, GR, HU, IE, IS, IT, LT, LU, LV, MC, NL, PL, PT, RO, SE, SI, SK, TR), OAPI (BF, BJ, CF, CG, CI, CM, GA, GN, GQ, GW, ML, MR, NE, SN, TD, TG).

Veröffentlicht:
— *mit internationalem Recherchenbericht*
— *mit dem Sequenzprotokollteil der Beschreibung in elektronischer Form getrennt veröffentlicht; auf Antrag vom Internationalen Büro erhältlich*

(88) Veröffentlichungsdatum des internationalen Recherchenberichts: 2. August 2007

Zur Erklärung der Zweibuchstaben-Codes und der anderen Abkürzungen wird auf die Erklärungen ("Guidance Notes on Codes and Abbreviations") am Anfang jeder regulären Ausgabe der PCT-Gazette verwiesen.

(54) Title: METHOD FOR ACTIVATING A NUCLEIC ACID FOR A POLYMERASE REACTION

(54) Bezeichnung: VERFAHREN ZUR AKTIVIERUNG EINER NUKLEINSÄURE FÜR EINE POLYMERASE-REAKTION

(57) Abstract: The present invention relates to a method for activating a nucleic acid for a polymerase reaction, involving the following steps: (a) heating a nucleic acid to a temperature of 55 °C to 80 °C, (b) cooling the nucleic acid to a temperature at which a polymerase does not show any significant drop in activity and (c) starting the polymerase reaction by adding a heat-labile polymerase to the nucleic acid.

(57) Zusammenfassung: Die vorliegende Erfindung betrifft ein Verfahren zur Aktivierung einer Nukleinsäure für eine Polymerase-Reaktion mit den Schritten: (a) Erwärmen einer Nukleinsäure auf eine Temperatur von 55°C bis 80°C, (b) Abkühlen der Nukleinsäure auf eine Temperatur, bei der eine Polymerase keine wesentliche Abnahme der Aktivität zeigt, und (c) Starten der Polymerase-Reaktion durch Zugabe einer hitzelabilen Polymerase zu der Nukleinsäure.

WO 2007/028833 A3

INTERNATIONAL SEARCH REPORT

International application No
PCT/EP2006/066223

A. CLASSIFICATION OF SUBJECT MATTER
INV. C12Q1/68

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
C12Q

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

EPO-Internal, WPI Data, PAJ, BIOSIS, MEDLINE

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	US 2004/161742 A1 (DEAN FRANK B ET AL) 19 August 2004 (2004-08-19) c.f. claims 1 - 22, 91, 127, 144,claims 1-22; examples 2,3,5-7	1-14
X	US 6 033 881 A (HIMMLER ET AL) 7 March 2000 (2000-03-07) c.f. claims 1, 6 and 17; column 6 - column 8; examples 2-12	1-14
X	SAMBROOK, FRITSCH, MANIATIS: "Molecular Cloning - A LABORATORY MANUAL" 1989, COLD SPRING HARBOR LABORATORY PRESS , USA , XP002361772 page 5.34 - page 5.49	1-4,7-13

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[X] Further documents are listed in the continuation of Box C.

[X] See patent family annex.

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"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
2 April 2007	13/04/2007

Name and mailing address of the ISA/
European Patent Office, P.B. 5818 Patentlaan 2
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Fax: (+31-70) 340-3016

Authorized officer
Botz, Jürgen

C(Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	WO 03/072809 A (COMMONWEALTH SCIENTIFIC AND INDUSTRIAL RESEARCH ORGANISATION; MOLLOY,) 4 September 2003 (2003-09-04) the whole document -----	1-14
Y	SUZUKI TOSHINORI ET AL: "Mechanistic studies on depurination and apurinic site chain breakage in oligodeoxyribonucleotides" NUCLEIC ACIDS RESEARCH, vol. 22, no. 23, 1994, pages 4997-5003, XP002361770 ISSN: 0305-1048 the whole document -----	1-14

INTERNATIONAL SEARCH REPORT

Information on patent family members

International application No
PCT/EP2006/066223

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Form PCT/ISA/210 (patent family annex) (April 2005)

INTERNATIONALER RECHERCHENBERICHT

Internationales Aktenzeichen
PCT/EP2006/066223

A. KLASSIFIZIERUNG DES ANMELDUNGSGEGENSTANDES
INV. C12Q1/68

Nach der Internationalen Patentklassifikation (IPC) oder nach der nationalen Klassifikation und der IPC

B. RECHERCHIERTE GEBIETE

Recherchierter Mindestprüfstoff (Klassifikationssystem und Klassifikationssymbole)
C12Q

Recherchierte, aber nicht zum Mindestprüfstoff gehörende Veröffentlichungen, soweit diese unter die recherchierten Gebiete fallen

Während der internationalen Recherche konsultierte elektronische Datenbank (Name der Datenbank und evtl. verwendete Suchbegriffe)

EPO-Internal, WPI Data, PAJ, BIOSIS, MEDLINE

C. ALS WESENTLICH ANGESEHENE UNTERLAGEN

Kategorie*	Bezeichnung der Veröffentlichung, soweit erforderlich unter Angabe der in Betracht kommenden Teile	Betr. Anspruch Nr.
X	US 2004/161742 A1 (DEAN FRANK B ET AL) 19. August 2004 (2004-08-19) c.f. claims 1 - 22, 91, 127, 144,Ansprüche 1-22; Beispiele 2,3,5-7	1-14
X	US 6 033 881 A (HIMMLER ET AL) 7. März 2000 (2000-03-07) c.f. claims 1, 6 and 17; Spalte 6 - Spalte 8; Beispiele 2-12	1-14
X	SAMBROOK, FRITSCH, MANIATIS: "Molecular Cloning - A LABORATORY MANUAL" 1989, COLD SPRING HARBOR LABORATORY PRESS , USA , XP002361772 Seite 5.34 - Seite 5.49	1-4,7-13
	-/--	

Weitere Veröffentlichungen sind der Fortsetzung von Feld C zu entnehmen Siehe Anhang Patentfamilie

* Besondere Kategorien von angegebenen Veröffentlichungen :
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Datum des Abschlusses der internationalen Recherche	Absendedatum des internationalen Recherchenberichts
2. April 2007	13/04/2007

Name und Postanschrift der Internationalen Recherchenbehörde	Bevollmächtigter Bediensteter
Europäisches Patentamt, P.B. 5818 Patentlaan 2 NL – 2280 HV Rijswijk Tel. (+31–70) 340–2040, Tx. 31 651 epo nl, Fax: (+31–70) 340–3016	Botz, Jürgen

Formblatt PCT/ISA/210 (Blatt 2) (April 2005)

C. (Fortsetzung) ALS WESENTLICH ANGESEHENE UNTERLAGEN

Kategorie*	Bezeichnung der Veröffentlichung, soweit erforderlich unter Angabe der in Betracht kommenden Teile	Betr. Anspruch Nr.
Y	WO 03/072809 A (COMMONWEALTH SCIENTIFIC AND INDUSTRIAL RESEARCH ORGANISATION; MOLLOY,) 4. September 2003 (2003-09-04) das ganze Dokument -----	1-14
Y	SUZUKI TOSHINORI ET AL: "Mechanistic studies on depurination and apurinic site chain breakage in oligodeoxyribonucleotides" NUCLEIC ACIDS RESEARCH, Bd. 22, Nr. 23, 1994, Seiten 4997-5003, XP002361770 ISSN: 0305-1048 das ganze Dokument -----	1-14

INTERNATIONALER RECHERCHENBERICHT

Angaben zu Veröffentlichungen, die zur selben Patentfamilie gehören

Internationales Aktenzeichen
PCT/EP2006/066223

Im Recherchenbericht angeführtes Patentdokument	Datum der Veröffentlichung	Mitglied(er) der Patentfamilie	Datum der Veröffentlichung
US 2004161742 A1	19-08-2004	US 2003118998 A1	26-06-2003
US 6033881 A	07-03-2000	AR 002470 A1	25-03-1998
		AT 402203 B	25-03-1997
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		CN 1650028 A	03-08-2005
		EP 1485505 A1	15-12-2004
		JP 2005518216 T	23-06-2005

Electronic Acknowledgement Receipt

EFS ID:	19114208
Application Number:	14067620
International Application Number:	
Confirmation Number:	4288
Title of Invention:	Nicking and Extension Amplification Reaction for the Exponential Amplification of Nucleic Acids
First Named Inventor/Applicant Name:	Brian K. Maples
Customer Number:	26161
Filer:	Ian J.S. Lodovice/mary florczak
Filer Authorized By:	Ian J.S. Lodovice
Attorney Docket Number:	30171-0025002 / ITI-001
Receipt Date:	23-MAY-2014
Filing Date:	30-OCT-2013
Time Stamp:	12:48:24
Application Type:	Utility under 35 USC 111(a)

Payment information:

Submitted with Payment	no

File Listing:

Document Number	Document Description	File Name	File Size(Bytes)/ Message Digest	Multi Part /.zip	Pages (if appl.)
1	Information Disclosure Statement (IDS) Form (SB08)	301700025002IDS.pdf	161782 d48d36e7d52c0456e871f517c8017cf7283c 4f48	no	2

Warnings:

Information:

This is not an USPTO supplied IDS fillable form					
2	Foreign Reference	EP2657350.pdf	1994869 9c5742a79a7499518ad7f5556239c0cfc5ed3116	no	111
Warnings:					
Information:					
3	Foreign Reference	EP2660333.pdf	1623318 5fe7669a342eca00e8eb95db559cc0bc8b5a209c	no	88
Warnings:					
Information:					
4	Foreign Reference	WO1994003635.pdf	1666807 d7ca6ed17d3d779beb846ce20edcf62f8f7c962c	no	51
Warnings:					
Information:					
5	Foreign Reference	WO2007028833.pdf	2850799 715cd152fed6642e8ee33705d893f20ae8c6cd4c	no	33
Warnings:					
Information:					
6	Non Patent Literature	D7aSequence.pdf	125123 82cca26f6ce721f5a2d7ce2d1ffc8f7a0d669f12	no	4
Warnings:					
Information:					
7	Non Patent Literature	NoticeofOpposition.pdf	1581844 e4b7ef886ca8b158b2315f15c44fee7fe74485bf	no	36
Warnings:					
Information:					
		Total Files Size (in bytes):	10004542		

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National Stage of an International Application under 35 U.S.C. 371
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New International Application Filed with the USPTO as a Receiving Office
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APPLICATION NUMBER	FILING OR 371(C) DATE	FIRST NAMED APPLICANT	ATTY. DOCKET NO./TITLE
14/067,620	10/30/2013	Brian K. Maples	30171-0025002 / ITI-001

CONFIRMATION NO. 4288

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PUBLICATION NOTICE

OC000000067546690

Title: Nicking and Extension Amplification Reaction for the Exponential Amplification of Nucleic Acids

Publication No. US-2014-0093883-A1
Publication Date: 04/03/2014

NOTICE OF PUBLICATION OF APPLICATION

The above-identified application will be electronically published as a patent application publication pursuant to 37 CFR 1.211, et seq. The patent application publication number and publication date are set forth above.

The publication may be accessed through the USPTO's publically available Searchable Databases via the Internet at www.uspto.gov. The direct link to access the publication is currently http://www.uspto.gov/patft/.

The publication process established by the Office does not provide for mailing a copy of the publication to applicant. A copy of the publication may be obtained from the Office upon payment of the appropriate fee set forth in 37 CFR 1.19(a)(1). Orders for copies of patent application publications are handled by the USPTO's Office of Public Records. The Office of Public Records can be reached by telephone at (703) 308-9726 or (800) 972-6382, by facsimile at (703) 305-8759, by mail addressed to the United States Patent and Trademark Office, Office of Public Records, Alexandria, VA 22313-1450 or via the Internet.

In addition, information on the status of the application, including the mailing date of Office actions and the dates of receipt of correspondence filed in the Office, may also be accessed via the Internet through the Patent Electronic Business Center at www.uspto.gov using the public side of the Patent Application Information and Retrieval (PAIR) system. The direct link to access this status information is currently http://pair.uspto.gov/. Prior to publication, such status information is confidential and may only be obtained by applicant using the private side of PAIR.

Further assistance in electronically accessing the publication, or about PAIR, is available by calling the Patent Electronic Business Center at 1-866-217-9197.

Office of Data Managment, Application Assistance Unit (571) 272-4000, or (571) 272-4200, or 1-888-786-0101

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

First Named Inventor	:	Brian K. Maples	Art Unit	:	1637
Serial No.	:	14/067,620	Examiner	:	Olayinka A. Oyeyemi
Filed	:	October 30, 2013	Conf. No.	:	4288
Title	:	NICKING AND EXTENSION AMPLIFICATION REACTION FOR THE EXPONENTIAL AMPLIFICATION OF NUCLEIC ACIDS			

Mail Stop Amendment
Commissioner for Patents
P.O. Box 1450
Alexandria, VA 22313-1450

RESPONSE TO RESTRICTION REQUIREMENT

Prior to examination, please amend the application as indicated on the following pages.

Amendments to the Claims:

This listing of claims replaces all prior versions and listings of claims in the application:

Listing of Claims:

1.-66. (Canceled)

67. (Currently Amended) A method ~~of amplifying~~, comprising:

preparing a mixture comprising:

(i) a target nucleic acid present in a sample obtained from an animal, the target nucleic acid having a target polynucleotide sequence,

(ii) a polymerase,

(iii) a nicking enzyme,

(iv) a first oligonucleotide comprising a nicking site and a nicking enzyme binding site, and

(v) a second oligonucleotide comprising a nicking site and a nicking enzyme binding site.

68. (Currently Amended) The method of claim 67, wherein the target polynucleotide sequence is amplified from steps comprising:

(a) forming a first duplex comprising the target polynucleotide sequence and the first oligonucleotide;

(b) extending, using the polymerase, the first oligonucleotide along the target polynucleotide sequence to form an extended first oligonucleotide comprising a sequence complementary to the second oligonucleotide;

(c) forming a second duplex comprising the second oligonucleotide and the extended first oligonucleotide;

(d) extending, using the polymerase, the second oligonucleotide along the extended first oligonucleotide to form a third duplex comprising an extended second oligonucleotide comprising a sequence complementary to the first oligonucleotide and a first double-stranded nicking enzyme binding site;

(e) nicking, with the nicking enzyme, ~~at~~ the first nicking site on the ~~first oligonucleotide~~ third duplex to produce a ~~first polynucleotide fragment~~ fourth duplex comprising the extended second oligonucleotide and a fragment of the extended first oligonucleotide; and

(f) extending, using the polymerase, the ~~first polynucleotide~~ fragment of the extended first oligonucleotide along the extended second oligonucleotide of the fourth duplex to produce a double-stranded nucleic acid product and a second double-stranded nicking enzyme binding site.

69. (Previously Presented) The method of claim 68, wherein the double-stranded nucleic acid product comprises:

i) a first strand and a second strand, wherein the first strand comprises a first polynucleotide sequence corresponding to the target polynucleotide sequence and the second strand comprises a second polynucleotide sequence complementary to the target polynucleotide sequence, and

ii) first and second double-stranded nicking sites spaced apart by the target polynucleotide sequence.

70. (Currently Amended) The method of claim 68, further comprising the steps of:

a) nicking, using the nicking enzyme, the first nicking site of the double-stranded nucleic acid product to ~~prepare a first polynucleotide fragment~~ produce a fifth duplex comprising a first polynucleotide sequence corresponding to the target polynucleotide sequence and a fragment of the first oligonucleotide, and nicking, using the nicking enzyme, the second nicking site of the double-stranded nucleic acid product to produce a ~~second polynucleotide fragment~~ sixth duplex comprising a second polynucleotide sequence complementary to the target polynucleotide sequence and a fragment of the second oligonucleotide;

b) extending, using the polymerase, ~~a portion of the first polynucleotide fragment~~ the fragment of the first oligonucleotide along the first polynucleotide sequence of the fifth duplex to

produce a first double stranded product comprising a copy of the nicking site and a copy of the first polynucleotide sequence ~~polynucleotide~~ and extending, using ~~a~~ the polymerase, ~~a portion of the second polynucleotide fragment~~ the fragment of the second oligonucleotide along the second polynucleotide sequence of the sixth duplex to produce a second double stranded product comprising a copy of the nicking site and a copy of the second polynucleotide sequence ~~polynucleotide~~; and

c) nicking, using the nicking enzyme, the copy of the nicking site of the first double stranded product ~~polynucleotide~~ to release a copy of the first polynucleotide sequence and nicking, using the nicking enzyme, the copy of the nicking site of the second double stranded product ~~polynucleotide~~ to release a copy of the second polynucleotide sequence.

71. (Previously Presented) The method of claim 67, wherein the animal is a human.

72. (Previously Presented) The method of claim 67, wherein the target nucleic acid is obtained from an animal pathogen.

73. (Previously Presented) The method of claim 72, wherein the animal pathogen is a single-stranded DNA virus, double-stranded DNA virus, or single-stranded RNA virus.

74. (Previously Presented) The method of claim 72, wherein the animal pathogen is a bacterium.

75. (Previously Presented) The method of claim 72, wherein the animal pathogen contains spores and the target polynucleotide is amplified from the spores without the need for lysis of the spores.

76. (Previously Presented) The method of claim 67, wherein the sample obtained from an animal is obtained from the blood, bone marrow, mucus, lymph, hard tissues (e.g. liver, spleen, kidney, lung or ovary), biopsies, sputum, saliva, tears, faeces or urine of the animal.

77. (Previously Presented) The method of claim 76, wherein the sample obtained from an animal is obtained from the mucus, sputum, or saliva of the animal.

78. (Previously Presented) The method of claim 67, wherein the target nucleic acid is double-stranded DNA.

79. (Previously Presented) The method of claim 67, wherein the target nucleic acid is single-stranded DNA.

80. (Previously Presented) The method of claim 67, wherein the target nucleic acid is RNA.

81. (Previously Presented) The method of claim 67, wherein the target nucleic acid is selected from the group consisting of genomic DNA, plasmid DNA, viral DNA, mitochondrial DNA, cDNA, synthetic double-stranded DNA and synthetic single-stranded DNA.

82. (Previously Presented) The method of claim 81, wherein the target nucleic acid is genomic DNA.

83. (Currently Amended) The method of claim ~~81~~ 67, wherein the target nucleic acid is viral DNA or viral RNA.

84. (Previously Presented) The method of claim 67, which is performed without an initial heat denaturation step.

85. (Previously Presented) The method of claim 67, wherein the nicking enzyme is Nt.BstNBI.

86. (Previously Presented) The method of claim 67, wherein the nicking enzyme does not nick within the target polynucleotide sequence.

87. (Previously Presented) The method of claim 67, which is performed without the use of temperature cycling.

88. (Previously Presented) The method of claim 67, which is performed at about 55°C-59°C.

89. (Previously Presented) The method of claim 67, which is performed at a constant temperature for about 1 to 20 minutes.

90. (Previously Presented) The method of claim 68, which is performed at a temperature higher than the melting temperature of the first oligonucleotide/target polynucleotide sequence complex.

91. (Previously Presented) The method of claim 67, further comprising detecting amplification product.

92. (Previously Presented) The method of claim 91, wherein the amplification product is detected by a detection method selected from the group consisting of gel electrophoresis, mass spectrometry, SYBR I fluorescence, SYBR II fluorescence, SYBR Gold, Pico Green, TOTO-3, intercalating dye detection, fluorescence resonance energy transfer (FRET), molecular beacon detection, surface capture, capillary electrophoresis, incorporation of labeled nucleotides to allow detection by capture, fluorescence polarization, and lateral flow capture, or a combination thereof.

93. (Previously Presented) The method of claim 67, wherein the target polynucleotide sequence is amplified 1E+9-fold or more in about five minutes.

94. (Currently amended) A method ~~of amplifying~~, comprising:

preparing a mixture comprising:

(i) a target nucleic acid present in a sample obtained from an animal, the target nucleic acid having a target polynucleotide sequence,

(ii) a polymerase,

(iii) a nicking enzyme,

(iv) a first oligonucleotide comprising a nicking site and a nicking enzyme binding site, and

(v) a second oligonucleotide comprising a nicking site and a nicking enzyme binding site; which method is performed without an initial heat denaturation step.

95. (Currently amended) A method ~~of amplifying~~, comprising:

preparing a mixture comprising:

(i) genomic DNA present in a sample obtained from an animal, the genomic DNA having a target polynucleotide sequence,

(ii) a polymerase,

(iii) a nicking enzyme,

(iv) a first oligonucleotide comprising a nicking site and a nicking enzyme binding site, and

(v) a second oligonucleotide comprising a nicking site and a nicking enzyme binding site; which method is performed without an initial heat denaturation step.

REMARKS

Upon entry of the present amendment, claims 67-95 will be pending. Claims 1-66 were previously canceled. Applicants have amended claims 67, 68, 70, 83, 94 and 95. Most of the amendments are simply to clarify scope and are supported throughout the specification. No new matter has been introduced by these amendments.

In response to the restriction requirement made in the action mailed March 4, 2014, Applicant elects the following species:

Group I. Origin of target Nucleic Acid: **c) ssRNA virus**; and

Group II. Target Nucleic Acid: **c) RNA**

for examination. As amended, claims 67-73, 76-77, 80 and 83-95 are readable thereon. The election is made without traverse.

Applicant asks that all claims be examined in view of the amendment to the claims.

Please apply any other necessary charges or credits to Deposit Account 06-1050, referencing the above attorney docket number.

Respectfully submitted,

Date: April 2, 2014_________________

/Ian J.S. Lodovice, Reg. No. 59,749/__________
Ian J. Lodovice
Reg. No. 59,749

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23199849.doc

Electronic Acknowledgement Receipt

EFS ID:	18655889
Application Number:	14067620
International Application Number:	
Confirmation Number:	4288
Title of Invention:	Nicking and Extension Amplification Reaction for the Exponential Amplification of Nucleic Acids
First Named Inventor/Applicant Name:	Brian K. Maples
Customer Number:	26161
Filer:	Ian J.S. Lodovice/Mary Florczak
Filer Authorized By:	Ian J.S. Lodovice
Attorney Docket Number:	30171-0025002 / ITI-001
Receipt Date:	02-APR-2014
Filing Date:	30-OCT-2013
Time Stamp:	17:19:42
Application Type:	Utility under 35 USC 111(a)

Payment information:

Submitted with Payment	no

File Listing:

Document Number	Document Description	File Name	File Size(Bytes)/ Message Digest	Multi Part /.zip	Pages (if appl.)
1		30171Response.pdf	91668 321a0d903919e523487605d7cc66dfc4142b55c8	yes	8

	Multipart Description/PDF files in .zip description		
	Document Description	**Start**	**End**
	Response to Election / Restriction Filed	1	1
	Claims	2	7
	Applicant Arguments/Remarks Made in an Amendment	8	8
Warnings:			
Information:			
	Total Files Size (in bytes):	91668	

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New Applications Under 35 U.S.C. 111
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New International Application Filed with the USPTO as a Receiving Office
If a new international application is being filed and the international application includes the necessary components for an international filing date (see PCT Article 11 and MPEP 1810), a Notification of the International Application Number and of the International Filing Date (Form PCT/RO/105) will be issued in due course, subject to prescriptions concerning national security, and the date shown on this Acknowledgement Receipt will establish the international filing date of the application.

PTO/SB/06 (09-11)
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PATENT APPLICATION FEE DETERMINATION RECORD Substitute for Form PTO-875	Application or Docket Number 14/067,620	Filing Date 10/30/2013	☐ To be Mailed

ENTITY: ☒ LARGE ☐ SMALL ☐ MICRO

APPLICATION AS FILED – PART I

FOR	(Column 1) NUMBER FILED	(Column 2) NUMBER EXTRA	RATE ($)	FEE ($)
☒ BASIC FEE (37 CFR 1.16(a), (b), or (c))	N/A	N/A	N/A	**280**
☐ SEARCH FEE (37 CFR 1.16(k), (i), or (m))	N/A	N/A	N/A	
☐ EXAMINATION FEE (37 CFR 1.16(o), (p), or (q))	N/A	N/A	N/A	
TOTAL CLAIMS (37 CFR 1.16(i))	minus 20 =	*	X $ =	
INDEPENDENT CLAIMS (37 CFR 1.16(h))	minus 3 =	*	X $ =	
☐ APPLICATION SIZE FEE (37 CFR 1.16(s))	If the specification and drawings exceed 100 sheets of paper, the application size fee due is $310 ($155 for small entity) for each additional 50 sheets or fraction thereof. See 35 U.S.C. 41(a)(1)(G) and 37 CFR 1.16(s).			
☐ MULTIPLE DEPENDENT CLAIM PRESENT (37 CFR 1.16(j))				
* If the difference in column 1 is less than zero, enter "0" in column 2.			TOTAL	**280**

APPLICATION AS AMENDED – PART II

AMENDMENT	04/02/2014	(Column 1) CLAIMS REMAINING AFTER AMENDMENT		(Column 2) HIGHEST NUMBER PREVIOUSLY PAID FOR	(Column 3) PRESENT EXTRA	RATE ($)	ADDITIONAL FEE ($)
	Total (37 CFR 1.16(i))	* 29	Minus	** 29	= 0	x $80 =	0
	Independent (37 CFR 1.16(h))	* 3	Minus	***3	= 0	x $420 =	0
	☐ Application Size Fee (37 CFR 1.16(s))						
	☐ FIRST PRESENTATION OF MULTIPLE DEPENDENT CLAIM (37 CFR 1.16(j))						
						TOTAL ADD'L FEE	**0**

AMENDMENT		(Column 1) CLAIMS REMAINING AFTER AMENDMENT		(Column 2) HIGHEST NUMBER PREVIOUSLY PAID FOR	(Column 3) PRESENT EXTRA	RATE ($)	ADDITIONAL FEE ($)
	Total (37 CFR 1.16(i))	*	Minus	**	=	X $ =	
	Independent (37 CFR 1.16(h))	*	Minus	***	=	X $ =	
	☐ Application Size Fee (37 CFR 1.16(s))						
	☐ FIRST PRESENTATION OF MULTIPLE DEPENDENT CLAIM (37 CFR 1.16(j))						
						TOTAL ADD'L FEE	

* If the entry in column 1 is less than the entry in column 2, write "0" in column 3.
** If the "Highest Number Previously Paid For" IN THIS SPACE is less than 20, enter "20".
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The "Highest Number Previously Paid For" (Total or Independent) is the highest number found in the appropriate box in column 1.

LIE
/GWENDOLYN MYERS/

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APPLICATION NO.	FILING DATE	FIRST NAMED INVENTOR	ATTORNEY DOCKET NO.	CONFIRMATION NO.
14/067,620	10/30/2013	Brian K. Maples	30171-0025002 / ITI-001	4288

26161 7590 03/04/2014
FISH & RICHARDSON P.C. (BO)
P.O. BOX 1022
MINNEAPOLIS, MN 55440-1022

EXAMINER	
OYEYEMI, OLAYINKA A	

ART UNIT	PAPER NUMBER
1637	

NOTIFICATION DATE	DELIVERY MODE
03/04/2014	ELECTRONIC

Please find below and/or attached an Office communication concerning this application or proceeding.

The time period for reply, if any, is set in the attached communication.

Notice of the Office communication was sent electronically on above-indicated "Notification Date" to the following e-mail address(es):

PATDOCTC@fr.com

PTOL-90A (Rev. 04/07)

Office Action Summary	Application No. 14/067,620	Applicant(s) MAPLES ET AL.	
	Examiner OLAYINKA OYEYEMI	Art Unit 1637	AIA (First Inventor to File) Status No

-- The MAILING DATE of this communication appears on the cover sheet with the correspondence address --

Period for Reply

A SHORTENED STATUTORY PERIOD FOR REPLY IS SET TO EXPIRE *2* MONTHS FROM THE MAILING DATE OF THIS COMMUNICATION.

- Extensions of time may be available under the provisions of 37 CFR 1.136(a). In no event, however, may a reply be timely filed after SIX (6) MONTHS from the mailing date of this communication.
- If NO period for reply is specified above, the maximum statutory period will apply and will expire SIX (6) MONTHS from the mailing date of this communication.
- Failure to reply within the set or extended period for reply will, by statute, cause the application to become ABANDONED (35 U.S.C. § 133). Any reply received by the Office later than three months after the mailing date of this communication, even if timely filed, may reduce any earned patent term adjustment. See 37 CFR 1.704(b).

Status

1)☐ Responsive to communication(s) filed on _____.
☐ A declaration(s)/affidavit(s) under **37 CFR 1.130(b)** was/were filed on _____.
2a)☐ This action is **FINAL**. 2b)☒ This action is non-final.
3)☐ An election was made by the applicant in response to a restriction requirement set forth during the interview on _____; the restriction requirement and election have been incorporated into this action.
4)☐ Since this application is in condition for allowance except for formal matters, prosecution as to the merits is closed in accordance with the practice under *Ex parte Quayle*, 1935 C.D. 11, 453 O.G. 213.

Disposition of Claims*

5)☒ Claim(s) *67-95* is/are pending in the application.
5a) Of the above claim(s) _____ is/are withdrawn from consideration.
6)☐ Claim(s) _____ is/are allowed.
7)☐ Claim(s) _____ is/are rejected.
8)☐ Claim(s) _____ is/are objected to.
9)☒ Claim(s) *67-95* are subject to restriction and/or election requirement.

* If any claims have been determined allowable, you may be eligible to benefit from the **Patent Prosecution Highway** program at a participating intellectual property office for the corresponding application. For more information, please see http://www.uspto.gov/patents/init_events/pph/index.jsp or send an inquiry to PPHfeedback@uspto.gov.

Application Papers

10)☐ The specification is objected to by the Examiner.
11)☐ The drawing(s) filed on _____ is/are: a)☐ accepted or b)☐ objected to by the Examiner.
Applicant may not request that any objection to the drawing(s) be held in abeyance. See 37 CFR 1.85(a).
Replacement drawing sheet(s) including the correction is required if the drawing(s) is objected to. See 37 CFR 1.121(d).

Priority under 35 U.S.C. § 119

12)☐ Acknowledgment is made of a claim for foreign priority under 35 U.S.C. § 119(a)-(d) or (f).
Certified copies:
a)☐ All b)☐ Some** c)☐ None of the:
1.☐ Certified copies of the priority documents have been received.
2.☐ Certified copies of the priority documents have been received in Application No. _____.
3.☐ Copies of the certified copies of the priority documents have been received in this National Stage application from the International Bureau (PCT Rule 17.2(a)).

** See the attached detailed Office action for a list of the certified copies not received.

Attachment(s)

1) ☐ Notice of References Cited (PTO-892)
2) ☐ Information Disclosure Statement(s) (PTO/SB/08a and/or PTO/SB/08b) Paper No(s)/Mail Date _____.
3) ☐ Interview Summary (PTO-413) Paper No(s)/Mail Date. _____ .
4) ☐ Other: _____.

DETAILED ACTION

Priority

1. This application is a CON of U.S. Patent Application No. 11/778,018 filed July 14, 2007.

Status of the claims

2. Claims 67-95 are pending.

Species Election

3. This application contains claims directed to more than one species of the generic invention. The species are independent or distinct because they are not obvious variants of each other based on the current record. For example, the method of producing a double stranded DNA amplification product of a target polynucleotide sequence from a pathogenic spore present in an animal sample using a mixture comprising polymerase, a nicking enzyme and two oligonucleotides, each comprising a nicking site and a nicking enzyme binding site, requires a different search and analysis from a method of producing a single stranded RNA amplification product of a virus target RNA polynucleotide sequence using a mixture comprising polymerase, a nicking enzyme and two oligonucleotides, each comprising a nicking site and a nicking enzyme binding site.

Applicant is required under 35 U.S.C. 121 to elect a single specie, for prosecution on the merits to which the claims shall be restricted if no generic claim is finally held to be allowable. Currently, claims 67-72, 76-77 and 84-95 are generic.

There is a search and/or examination burden for the patentably distinct species as the searches are not coextensive and it would burdensome to search and examine all of the claims:

The species are Groups I-II. Make an election for each group indicated as set forth below:

I. Origin of the target Nucleic Acid, elect one of the following groups

a) ssDNA virus (claims 73, 81 and 83);

b) dsDNA virus (claims 73, 81 and 83);

c) ssRNA virus(claim 73);

d) Bacterium (claim 74);

e) pathogen containing spores (claim 75).

II. Target Nucleic Acid, elect one of the following groups

a) double stranded DNA;

b) single stranded DNA;

c) RNA.

4. Applicant is advised that a reply to this requirement must include an identification of the species that is elected consonant with this requirement, and a listing of all claims readable thereon, including any claims subsequently added. An argument that a claim is allowable or that all claims are generic is considered nonresponsive unless accompanied by an election.

Upon the allowance of a generic claim, applicant will be entitled to consideration of claims to additional species which depend from or otherwise require all the limitations of an allowable generic claim as provided by 37 CFR 1.141. If claims are added after the election, applicant must indicate which are readable upon the elected species. MPEP § 809.02(a).

5. Applicant is advised that the reply to this requirement to be complete must include (i) an election of a species to be examined even though the requirement may be traversed (37 CFR 1.143) and (ii) identification of the claims encompassing the elected species, including any claims subsequently added. An argument that a claim is allowable or that all claims are generic is considered nonresponsive unless accompanied by an election.

6. The election of species may be made with or without traverse. To preserve a right to petition, the election must be made with traverse. If the reply does not distinctly and specifically point out supposed

errors in the election of species requirement, the election shall be treated as an election without traverse. Traversal must be presented at the time of election in order to be considered timely. Failure to timely traverse the requirement will result in the loss of right to petition under 37 CFR 1.144. If claims are added after the election, applicant must indicate which of these claims are readable on the elected species or grouping of patentably indistinct species.

Should applicant traverse on the ground that the species, or groupings of patentably indistinct species from which election is required, are not patentably distinct, applicant should submit evidence or identify such evidence now of record showing them to be obvious variants or clearly admit on the record that this is the case. In either instance, if the examiner finds one of the species unpatentable over the prior art, the evidence or admission may be used in a rejection under 35 U.S.C. 103(a) of the other species.

7. Applicant is reminded that upon the cancellation of claims to a non-elected invention, the inventorship must be amended in compliance with 37 CFR 1.48(b) if one or more of the currently named inventors is no longer an inventor of at least one claim remaining in the application. Any amendment of inventorship must be accompanied by a request under 37 CFR 1.48(b) and by the fee required under 37 CFR 1.17(i).

Correspondence

8. Any inquiry concerning this communication or earlier communications from the examiner should be directed to OLAYINKA OYEYEMI whose telephone number is (571)270-5956. The examiner can normally be reached on M -Thurs 9-3 pm EST.

If attempts to reach the examiner by telephone are unsuccessful, the examiner's supervisor, Gary Benzion can be reached on 571-272-0782. The fax phone number for the organization where this application or proceeding is assigned is 571-273-8300.

Information regarding the status of an application may be obtained from the Patent Application Information Retrieval (PAIR) system. Status information for published applications may be obtained from either Private PAIR or Public PAIR. Status information for unpublished applications is available through

Private PAIR only. For more information about the PAIR system, see http://pair-direct.uspto.gov. Should you have questions on access to the Private PAIR system, contact the Electronic Business Center (EBC) at 866-217-9197 (toll-free). If you would like assistance from a USPTO Customer Service Representative or access to the automated information system, call 800-786-9199 (IN USA OR CANADA) or 571-272-1000.

/OLAYINKA OYEYEMI/
Examiner, Art Unit 1637

/Angela M. Bertagna/
Primary Examiner, Art Unit 1637

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DEC 30 2013

OFFICE OF PETITIONS

FISH & RICHARDSON P.C. (BO)
P.O. BOX 1022
MINNEAPOLIS MN 55440-1022

Doc Code: TRACK1.GRANT

Decision Granting Request for Prioritized Examination (Track I or After RCE)	Application No.: 14/067,620

1. THE REQUEST FILED October 30, 2013 IS **GRANTED**.

The above-identified application has met the requirements for prioritized examination
- A. ☒ for an original nonprovisional application (Track I).
- B. ☐ for an application undergoing continued examination (RCE).

2. **The above-identified application will undergo prioritized examination.** The application will be accorded special status throughout its entire course of prosecution until one of the following occurs:

- A. filing a **petition for extension of time** to extend the time period for filing a reply;
- B. filing an **amendment to amend the application to contain more than four independent claims, more than thirty total claims**, or a multiple dependent claim;
- C. filing a **request for continued examination**;
- D. filing a notice of appeal;
- E. filing a request for suspension of action;
- F. mailing of a notice of allowance;
- G. mailing of a final Office action;
- H. completion of examination as defined in 37 CFR 41.102; or
- I. abandonment of the application.

Telephone inquiries with regard to this decision should be directed to Brian W. Brown at 571-272-5338.

/Brian W. Brown/
[*Signature*]

Petitions Examiner, Office of Petitions
(Title)

U.S. Patent and Trademark Office
PTO-2298 (Rev. 02-2012)

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APPLICATION NUMBER	FILING or 371(c) DATE	GRP ART UNIT	FIL FEE REC'D	ATTY.DOCKET.NO	TOT CLAIMS	IND CLAIMS
14/067,620	10/30/2013	1637	2620	30171-0025002 / ITI-001	29	3

CONFIRMATION NO. 4288

26161
FISH & RICHARDSON P.C. (BO)
P.O. BOX 1022
MINNEAPOLIS, MN 55440-1022

UPDATED FILING RECEIPT

OC000000065655376

Date Mailed: 12/27/2013

Receipt is acknowledged of this non-provisional patent application. The application will be taken up for examination in due course. Applicant will be notified as to the results of the examination. Any correspondence concerning the application must include the following identification information: the U.S. APPLICATION NUMBER, FILING DATE, NAME OF APPLICANT, and TITLE OF INVENTION. Fees transmitted by check or draft are subject to collection. Please verify the accuracy of the data presented on this receipt. **If an error is noted on this Filing Receipt, please submit a written request for a Filing Receipt Correction. Please provide a copy of this Filing Receipt with the changes noted thereon. If you received a "Notice to File Missing Parts" for this application, please submit any corrections to this Filing Receipt with your reply to the Notice. When the USPTO processes the reply to the Notice, the USPTO will generate another Filing Receipt incorporating the requested corrections**

Inventor(s)

Brian K. Maples, Lake Forest, CA;
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Ionian Technologies, Inc., San Diego, CA

Assignment For Published Patent Application

Ionian Technologies, Inc., San Diego, CA

Power of Attorney: The patent practitioners associated with Customer Number 26161

Domestic Priority data as claimed by applicant

This application is a CON of 11/778,018 07/14/2007

Foreign Applications for which priority is claimed (You may be eligible to benefit from the **Patent Prosecution Highway** program at the USPTO. Please see http://www.uspto.gov for more information.) - None.
Foreign application information must be provided in an Application Data Sheet in order to constitute a claim to foreign priority. See 37 CFR 1.55 and 1.76.

If Required, Foreign Filing License Granted: 11/21/2013
The country code and number of your priority application, to be used for filing abroad under the Paris Convention, is **US 14/067,620**
Projected Publication Date: 04/03/2014

Non-Publication Request: No

Early Publication Request: No
Title

Nicking and Extension Amplification Reaction for the Exponential Amplification of Nucleic Acids

Preliminary Class

435

Statement under 37 CFR 1.55 or 1.78 for AIA (First Inventor to File) Transition Applications: No

PROTECTING YOUR INVENTION OUTSIDE THE UNITED STATES

Since the rights granted by a U.S. patent extend only throughout the territory of the United States and have no effect in a foreign country, an inventor who wishes patent protection in another country must apply for a patent in a specific country or in regional patent offices. Applicants may wish to consider the filing of an international application under the Patent Cooperation Treaty (PCT). An international (PCT) application generally has the same effect as a regular national patent application in each PCT-member country. The PCT process **simplifies** the filing of patent applications on the same invention in member countries, but **does not result** in a grant of "an international patent" and does not eliminate the need of applicants to file additional documents and fees in countries where patent protection is desired.

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Applicants also are advised that in the case of inventions made in the United States, the Director of the USPTO must issue a license before applicants can apply for a patent in a foreign country. The filing of a U.S. patent application serves as a request for a foreign filing license. The application's filing receipt contains further information and guidance as to the status of applicant's license for foreign filing.

Applicants may wish to consult the USPTO booklet, "General Information Concerning Patents" (specifically, the section entitled "Treaties and Foreign Patents") for more information on timeframes and deadlines for filing foreign patent applications. The guide is available either by contacting the USPTO Contact Center at 800-786-9199, or it can be viewed on the USPTO website at http://www.uspto.gov/web/offices/pac/doc/general/index.html.

For information on preventing theft of your intellectual property (patents, trademarks and copyrights), you may wish to consult the U.S. Government website, http://www.stopfakes.gov. Part of a Department of Commerce initiative, this website includes self-help "toolkits" giving innovators guidance on how to protect intellectual property in specific countries such as China, Korea and Mexico. For questions regarding patent enforcement issues, applicants may call the U.S. Government hotline at 1-866-999-HALT (1-866-999-4258).

LICENSE FOR FOREIGN FILING UNDER

Title 35, United States Code, Section 184

Title 37, Code of Federal Regulations, 5.11 & 5.15

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PATENT APPLICATION FEE DETERMINATION RECORD

Substitute for Form PTO-875

Application or Docket Number
14/067,620

APPLICATION AS FILED - PART I

FOR	NUMBER FILED (Column 1)	NUMBER EXTRA (Column 2)	SMALL ENTITY RATE($)	SMALL ENTITY FEE($)	OR	OTHER THAN SMALL ENTITY RATE($)	OTHER THAN SMALL ENTITY FEE($)
BASIC FEE (37 CFR 1.16(a), (b), or (c))	N/A	N/A	N/A			N/A	280
SEARCH FEE (37 CFR 1.16(k), (i), or (m))	N/A	N/A	N/A			N/A	600
EXAMINATION FEE (37 CFR 1.16(o), (p), or (q))	N/A	N/A	N/A			N/A	720
TOTAL CLAIMS (37 CFR 1.16(i))	29 minus 20 =	* 9			OR	x 80 =	720
INDEPENDENT CLAIMS (37 CFR 1.16(h))	3 minus 3 =	*				x 420 =	0.00
APPLICATION SIZE FEE (37 CFR 1.16(s))	If the specification and drawings exceed 100 sheets of paper, the application size fee due is $310 ($155 for small entity) for each additional 50 sheets or fraction thereof. See 35 U.S.C. 41(a)(1)(G) and 37 CFR 1.16(s).						0.00
MULTIPLE DEPENDENT CLAIM PRESENT (37 CFR 1.16(j))							0.00
			TOTAL			TOTAL	2320

* If the difference in column 1 is less than zero, enter "0" in column 2.

APPLICATION AS AMENDED - PART II

AMENDMENT A	CLAIMS REMAINING AFTER AMENDMENT (Column 1)		HIGHEST NUMBER PREVIOUSLY PAID FOR (Column 2)	PRESENT EXTRA (Column 3)	SMALL ENTITY RATE($)	SMALL ENTITY ADDITIONAL FEE($)	OR	OTHER THAN SMALL ENTITY RATE($)	OTHER THAN SMALL ENTITY ADDITIONAL FEE($)
Total (37 CFR 1.16(i))	*	Minus	**	=	x =		OR	x =	
Independent (37 CFR 1.16(h))	*	Minus	***	=	x =		OR	x =	
Application Size Fee (37 CFR 1.16(s))									
FIRST PRESENTATION OF MULTIPLE DEPENDENT CLAIM (37 CFR 1.16(j))							OR		
					TOTAL ADD'L FEE		OR	TOTAL ADD'L FEE	

AMENDMENT B	CLAIMS REMAINING AFTER AMENDMENT (Column 1)		HIGHEST NUMBER PREVIOUSLY PAID FOR (Column 2)	PRESENT EXTRA (Column 3)	SMALL ENTITY RATE($)	SMALL ENTITY ADDITIONAL FEE($)	OR	OTHER THAN SMALL ENTITY RATE($)	OTHER THAN SMALL ENTITY ADDITIONAL FEE($)
Total (37 CFR 1.16(i))	*	Minus	**	=	x =		OR	x =	
Independent (37 CFR 1.16(h))	*	Minus	***	=	x =		OR	x =	
Application Size Fee (37 CFR 1.16(s))									
FIRST PRESENTATION OF MULTIPLE DEPENDENT CLAIM (37 CFR 1.16(j))							OR		
					TOTAL ADD'L FEE		OR	TOTAL ADD'L FEE	

* If the entry in column 1 is less than the entry in column 2, write "0" in column 3.
** If the "Highest Number Previously Paid For" IN THIS SPACE is less than 20, enter "20".
*** If the "Highest Number Previously Paid For" IN THIS SPACE is less than 3, enter "3".
The "Highest Number Previously Paid For" (Total or Independent) is the highest found in the appropriate box in column 1.

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

Applicant	:	Ionian Technologies Inc.	Art Unit	:	1637
Serial No.	:	14/067,620	Examiner	:	Unknown
Filed	:	October 30, 2013	Conf. No.	:	4288
Title	:	NICKING AND EXTENSION AMPLIFICATION REACTION FOR THE EXPONENTIAL AMPLIFICATION OF NUCLEIC ACIDS			

Commissioner for Patents
P.O. Box 1450
Alexandria, VA 22313-1450

RESPONSE TO NOTICE TO FILE CORRECTED APPLICATION PAPERS DATED DECEMBER 16, 2013

The applicant as a large entity submits:

☒ Replacement drawings (19 sheets) in compliance with 37 CFR §1.84.

☒ A substitute specification in compliance with 37 CFR §1.52. No new matter has been added.

The applicant understands that this perfects the application and no additional papers or filing fees are required.

Please apply any necessary charges or credits to Deposit Account 06-1050, referencing the above attorney docket number.

Respectfully submitted,

Date: December 18, 2013

/Ian J.S. Lodovice, Reg. No. 59,749/
Ian J. Lodovice
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23136160.doc

NICKING AND EXTENSION AMPLIFICATION REACTION FOR THE EXPONENTIAL AMPLIFICATION OF NUCLEIC ACIDS

Related Applications

This application is a continuation of U.S. Application Serial No. 11/778,018, filed July 14, 2007, the entire contents of which are hereby incorporated.

Field of the Invention

The invention is in general directed to the rapid exponential amplification of short DNA or RNA sequences at a constant temperature.

Background

The field of *in vitro* diagnostics is quickly expanding as the need for systems that can rapidly detect the presence of harmful species or determine the genetic sequence of a region of interest is increasing exponentially. Current molecular diagnostics focus on the detection of biomarkers and include small molecule detection, immuno-based assays, and nucleic acid tests. The built-in specificity between two complementary nucleic acid strands allows for fast and specific recognition using unique DNA or RNA sequences, the simplicity of which makes a nucleic acid test an attractive prospect. Identification of bacterial and viral threat agents, genetically modified food products, and single nucleotide polymorphisms for disease management are only a few areas where the advancement of these molecular diagnostic tools becomes extremely advantageous. To meet these growing needs, nucleic acid amplification technologies have been developed and tailored to these needs of specificity and sensitivity.

Historically, the most common amplification technique is the polymerase chain reaction (PCR), which has in many cases become the gold standard for detection methods because of its reliability and specificity. This technique requires the cycling of temperatures to proceed through the steps of denaturation of the dsDNA, annealing of short oligonucleotide primers, and extension of the primer along the template by a thermostable polymerase. Though many new advances in engineering have successfully shortened these reaction times to 20-30 minutes, there is still a steep power requirement to meet the needs of these thermocycling units.

Various isothermal amplification techniques have been developed to circumvent the need for temperature cycling. From this demand, both DNA and RNA isothermal amplification technologies have emerged.

Transcription-Mediated Amplification (TMA) employs a reverse transcriptase with RNase activity, an RNA polymerase, and primers with a promoter sequence at the 5'end. The reverse transcriptase synthesizes cDNA from the primer, degrades the RNA target, and synthesizes the second strand after the reverse primer binds. RNA polymerase then binds to the promoter region of the dsDNA and transcribes new RNA transcripts which can serve as templates for further reverse transcription. The reaction can produce a billion fold amplification in 20-30 minutes. This system is not as robust as other DNA amplification techniques and is therefore, not a field-deployable test due to the ubiquitous presence of RNAases outside of a sterile laboratory. This amplification technique is very similar to Self-Sustained Sequence Replication (3SR) and Nucleic Acid Sequence Based Amplification (NASBA), but varies in the enzymes employed.

Single Primer Isothermal Amplification (SPIA) also involves multiple polymerases and RNaseH. First, a reverse transcriptase extends a chimeric primer along an RNA target. RNaseH degrades the RNA target and allows a DNA polymerase to synthesize the second strand of cDNA. RNaseH then degrades a portion of the chimeric primer to release a portion of the cDNA and open a binding site for the next chimeric primer to bind and the amplification process proceeds through the cycle again. The linear amplification system can amplify very low levels of RNA target in roughly 3.5 hrs.

The Q-Beta replicase system is a probe amplification method. A probe region complementary to the target of choice is inserted into MDV-l RNA, a naturally occurring template for Q-Beta replicase. Q-Beta replicates the MDV -1 plasmid so that the synthesized product is itself a template for Q-Beta replicase, resulting in exponential amplification as long as the there is excess replicase to template. Because the Q-Beta replication process is so sensitive and can amplify whether the target is present or not, multiple wash steps are required to purge the sample of non-specifically bound replication plasmids. The exponential amplification takes approximately 30 minutes; however, the total time including all wash steps is approximately 4 hours.

Numerous isothermal DNA amplification technologies have been developed as well. Rolling circle amplification (RCA) was developed based on the natural replication of plasmids and viruses. A primer extends along a circular template resulting in the synthesis of a single-stranded tandem repeat. Capture, washing, and ligation steps are necessary to preferentially circularize the template in the presence of target and reduce background amplification. Ramification amplification (RAM) adds cascading primers for additional geometric amplification. This technique involves amplification of non-specifically sized strands that are either double or single-stranded.

Helicase-dependent amplification (HDA) takes advantage of a thermostable helicase (Tte-UvrD) to unwind dsDNA to create single-strands that are then available for hybridization and extension of primers by polymerase. The thermostable HDA method does not require the accessory proteins that the non-thermostable HDA requires. The reaction can be performed at a single temperature, though an initial heat denaturation to bind the primers generates more product. Reaction times are reported to be over 1 hour to amplify products 70-120 base pairs in length.

Loop mediated amplification (LAMP) is a sensitive and specific isothermal amplification method that employs a thermostable polymerase with strand displacement capabilities and four or more primers. The primers are designed to anneal consecutively along the target in the forward and reverse direction. Extension of the outer primers displaces the extended inner primers to release single strands. Each primer is designed to have hairpin ends that, once displaced, snap into a hairpin to facilitate self-priming and further polymerase extension. Additional loop primers can decrease the amplification time, but complicates the reaction mixture. Overall, LAMP is a difficult amplification method to multiplex, that is, to amplify more than one target sequence at a time, although it is reported to be extremely specific due to the multiple primers that must anneal to the target to further the amplification process. Though the reaction proceeds under isothermal conditions, an initial heat denaturation step is required for double-stranded targets. Amplification proceeds in 25 to 50 minutes and yields a ladder pattern of various length products.

Strand displacement amplification (SDA) was developed by Walker et.al. in 1992. This amplification method uses two sets of primers, a strand displacing polymerase, and a restriction

endonuclease. The bumper primers serve to displace the initially extended primers to create a single-strand for the next primer to bind. A restriction site is present in the 5' region of the primer. Thiol-modified nucleotides are incorporated into the synthesized products to inhibit cleavage of the synthesized strand. This modification creates a nick site on the primer side of the strand, which the polymerase can extend. This approach requires an initial heat denaturation step for double-stranded targets. The reaction is then run at a temperature below the melting temperature of the double-stranded target region. Products 60 to 100 bases in length are usually amplified in 30-45 minutes using this method.

These and other amplification methods are discussed in, for example, VanNess, J, et al., PNAS 2003. Vol. 100, no 8, p 4504-4509; Tan, E., et al., Anal. Chem. 2005, 77, 7984-7992; Lizard, P., et al., Nature Biotech. 1998, 6, 1197-1202; Notomi, T., et al., NAR 2000,28,12, e63; and Kurn, N., et al., Clin. Chem. 2005, 51:10,1973-1981. Other references for these general amplification techniques include, for example, U.S. Patent Serial Nos. 7112423; 5455166; 5712124; 5744311; 5916779; 5556751;5733733; 5834202;5354668; 5591609; 5614389; 5942391; and U.S. patent publication numbers US20030082590; US20030138800; US20040058378; and US20060154286.

There is a need for a quicker method of amplification of single-stranded and double-stranded nucleic acid target sequences that can be performed without temperature cycling and that is suitable for shorter target sequences.

Summary

Provided herein are methods of amplifying nucleic acid target sequences that rely on nicking and extension reactions and amplify shorter sequences in a quicker timeframe than traditional amplification reactions, such as, for example, strand displacement amplification reactions. Embodiments of the invention include, for example, reactions that use only two templates to prime, one or two nicking enzymes, and a polymerase, under isothermal conditions. In exemplary embodiments, the polymerase and the nicking enzyme are thermophilic, and the reaction temperature is significantly above the melting temperature of the hybridized target region. The nicking enzyme nicks only one strand in a double-stranded duplex, so that incorporation of modified nucleotides is not necessary as it is in strand displacement. An initial

heat denaturation step is not required for the methods of the present invention. Due to the simplicity of the reaction, in exemplary embodiments, the reaction is very easy to perform and can amplify 20-30mer products 10^8 to 10^{10} fold from genomic DNA in 2.5 to 10 minutes. Furthermore, in other exemplary embodiments, the method is able to amplify RNA without a separate reverse transcription step.

Thus, provided in a first embodiment of the present invention is a method for amplifying a double-stranded nucleic acid target sequence, comprising contacting a target DNA molecule comprising a double-stranded target sequence having a sense strand and an antisense strand, with a forward template and a reverse template, wherein said forward template comprises a nucleic acid sequence comprising a recognition region at the 3' end that is complementary to the 3' end of the target sequence antisense strand; a nicking enzyme site upstream of said recognition region, and a stabilizing region upstream of said nicking enzyme site; said reverse template comprises a nucleotide sequence comprising a recognition region at the 3' end that is complementary to the 3' end of the target sequence sense strand, a nicking enzyme site upstream of said recognition region, and a stabilizing region upstream of said nicking enzyme site; providing a first nicking enzyme that is capable of nicking at the nicking enzyme site of said forward template, and does not nick within said target sequence; providing a second nicking enzyme that is capable of nicking at the nicking enzyme site of said reverse template and does not nick within said target sequence; and providing a DNA polymerase; under conditions wherein amplification is performed by multiple cycles of said polymerase extending said forward and reverse templates along said target sequence producing a double-stranded nicking enzyme site, and said nicking enzymes nicking at said nicking enzyme sites, producing an amplification product.

In certain embodiments of the invention, the DNA polymerase is a thermophilic polymerase. In other examples of the invention, the polymerase and said nicking enzymes are stable at temperatures up to 37°C, 42°C, 60°C, 65°C, 70°C, 75°C, 80°C, or 85°C. In certain embodiments, the polymerase is stable up to 60°C. The polymerase may, for example, be selected from the group consisting of Bst (large fragment), 9°N, VentR ® (exo-) DNA Polymerase, Therminator, and Therminator II.

The nicking enzyme may, for example, nick upstream of the nicking enzyme binding site,

or, in exemplary embodiments, the nicking enzyme may nick downstream of the nicking enzyme binding site. In certain embodiments, the forward and reverse templates comprise nicking enzyme sites recognized by the same nicking enzyme and said first and said second nicking enzyme are the same. The nicking enzyme may, for example, be selected from the group consisting of Nt.BspQI, Nb.BbvCi, Nb.BsmI, Nb.BsrDI, Nb.BtsI, Nt.AlwI, Nt.BbvCI, Nt.BstNBI, Nt.CviPII, Nb.BpulOI, and Nt.Bpul0I.

In certain aspects of the present invention, the target sequence comprises from 1 to 5 nucleotides more than the sum of the nucleotides of said forward template recognition region and said reverse template recognition region.

The DNA molecule may be, for example, genomic DNA. The DNA molecule may be, for example, selected from the group consisting of plasmid, mitochondrial, and viral DNA. In certain embodiments, the forward template is provided at the same concentration as the reverse template. In other examples, the forward template is provided at a ratio to the reverse template at the range of ratios of 1: 100 to 100:1.

In other examples of the invention, the method further comprises the use of a second polymerase. The amplification may be, for example, conducted at a constant temperature. This temperature may be, for example, between 54°C and 60°C. As to the length of time for the reaction to take place, in certain examples, the amplification reaction is held at constant temperature for 1 to 10 minutes.

The present invention further comprises detecting the amplification product, for example, by a method selected from the group consisting of gel electrophoresis, mass spectrometry, SYBR I fluorescence, SYBR II fluorescence, SYBR Gold, Pico Green, TOTO-3, intercalating dye detection, FRET, molecular beacon detection, surface capture, capillary electrophoresis, incorporation of labeled nucleotides to allow detection by capture, fluorescence polarization, and lateral flow capture. The amplification products may be, for example, detected using a solid surface method, for example, where at least one capture probe is immobilized on the solid surface that binds to the amplified sequence.

The present invention may be used for multiplex amplification. Thus, for example, in certain embodiments of the present invention at least two target sequences are capable of being amplified. By "capable of being amplified" is meant the amplification reaction comprises the

appropriate templates and enzymes to amplify at least two target sequences. Thus, for example, the amplification reaction may be prepared to detect at least two target sequences, but only one of the target sequences may actually be present in the sample being tested, such that both sequences are capable of being amplified, but only one sequence is. Or, where two target sequences are present, the amplification reaction may result in the amplification of both of the target sequences. The multiplex amplification reaction may result in the amplification of one, some, or all, of the target sequences for which it comprises the appropriate templates and enzymes.

At least one of the templates, for example, may comprise a spacer, a blocking group, or a modified nucleotide.

Also provided as an embodiment of the present invention is a method for amplifying a single-stranded nucleic acid target sequence, comprising contacting a target nucleic acid comprising a single-stranded target sequence with a reverse template, wherein said reverse template comprises a nucleotide sequence comprising a recognition region at the 3' end that is complementary to the 3' end of the target sequence, a nicking enzyme site upstream of said recognition region, and a stabilizing region upstream of said nicking enzyme site; providing a first nicking enzyme that is capable of nicking at the nicking enzyme site of said reverse template, and does not nick within said target sequence; providing a DNA polymerase under conditions wherein said polymerase extends said reverse template along said target sequence; contacting said extended reverse template with a forward template, wherein said forward template comprises comprising a recognition region at the 3' end that is identical to the 5' end of the target sequence a nicking enzyme site upstream of said recognition region, and a stabilizing region upstream of said nicking enzyme site; providing a second nicking enzyme that is capable of nicking at the nicking enzyme site of said forward template and does not nick within said target sequence; under conditions wherein amplification is performed by multiple cycles of said polymerase extending said forward and reverse templates along said target sequence producing a double-stranded nicking enzyme site, and said nicking enzymes nicking at said nicking enzyme sites, producing an amplification product.

Those of ordinary skill in the art understand that the examples presented herein relating to the amplification of a double-stranded nucleic acid target sequence and the detection of the

amplified product also apply to the amplification of a single-stranded nucleic acid target sequence and the detection of the amplified product. Further, in examples of the present invention, the target sequence may be, for example, RNA, for example, but not limited to, messenger RNA, viral RNA, microRNA, a microRNA precursor, or siRNA. In exemplary embodiments of the present invention, the polymerase comprises reverse transcription activity. In yet other examples of the present invention, the target sequence is DNA, such as, for example, genomic DNA, or for example, the target sequence is selected from the group consisting of plasmid, mitochondrial, and viral nucleic acid.

Where the method may comprise the use of more than one polymerase, in exemplary embodiments at least one of the polymerases comprises reverse transcriptase activity.

In other embodiments of the present invention, a set of oligonucleotide templates is provided, comprising a first template for nucleic acid amplification, comprising a recognition region at the 3' end that is complementary to the 3' end of a target sequence antisense strand; a nicking enzyme site upstream of said recognition region; and a stabilizing region upstream of said nicking enzyme site; and a second template for nucleic acid amplification, comprising a recognition region at the 3' end that is identical to the 5' of said target sequence antisense strand; a nicking enzyme site upstream of said recognition region; and a stabilizing region upstream of said nicking enzyme site; wherein said target sequence comprises from 1 to 5 spacer bases between said 3' end of the antisense strand and said 5' end of said antisense strand that do not bind to either template.

In yet other embodiments, a kit is provided for following the methods of the present invention for nucleic acid amplification, comprising a DNA polymerase; a first template for nucleic acid amplification, comprising a recognition region at the 3' end that is complementary to the 3' end of a target sequence antisense strand; a nicking enzyme site upstream of said recognition region; and a stabilizing region upstream of said nicking enzyme site; a second template for nucleic acid amplification, comprising a recognition region at the 3' end that is complementary to the 3' end of a target sequence sense strand; a nicking enzyme site upstream of said recognition region; and a stabilizing region upstream of said nicking enzyme site; one or two thermostable nicking enzymes, wherein either one enzyme is capable of nicking at the nicking enzyme site of said first and said second templates, or a first enzyme is capable of

nicking at the nicking enzyme site of said first primer and a second enzyme is capable of nicking at the enzyme site of said second primer.

The kit may, for example, provide said polymerase, nicking enzymes, and templates in a container. The kit may provide, for example, said polymerase, nicking enzymes, and templates in two containers. In certain examples, the polymerase and nicking enzymes are in a first container, and said templates are in a second container. In certain examples, the polymerase and nicking enzymes are lyophilized. The kit may, for example, further comprise instructions for following the amplification methods of the present invention. The kit may, for example, further comprise a cuvette. The kit may, for example, further comprise a lateral flow device or dipstick. The lateral flow device or dipstick may, for example, further comprise a capture probe, wherein said capture probe binds to amplified product. The kit may, for example, further comprise a detector component selected from the group consisting of a fluorescent dye, colloidal gold particles, latex particles, a molecular beacon, and polystyrene beads. In other examples, at least one of the templates of the kit comprises a spacer, blocking group, or a modified nucleotide.

Deoxynucleoside triphosphates (dNTPs) are included in the amplification reaction. One or more of the dNTPs may be modified, or labeled, as discussed herein. Nucleotides are designated as follows. A ribonucleoside triphosphate is referred to as NTP or rNTP; N can be A, G, C, U or m5U to denote specific ribonucleotides. Deoxynucleoside triphosphate substrates are indicated as dNTPs, where N can be A, G, C, T, or U. Throughout the text, monomeric nucleotide subunits may be denoted as A, G, C, or T with no particular reference to DNA or RNA.

In another embodiment, a method is provided for nucleic acid amplification comprising forming a mixture of a target nucleic acid comprising a double-stranded target sequence having a sense strand and an antisense strand; a forward template comprising a nucleic acid sequence comprising a recognition region at the 3' end that is complementary to the 3' end of the target sequence antisense strand; a nicking enzyme site upstream of said recognition region, and a stabilizing region upstream of said nicking enzyme site; a reverse template comprising a nucleotide sequence comprising a recognition region at the 3' end that is complementary to the 3' end of the target sequence sense strand, a nicking enzyme site upstream of said recognition region and a stabilizing region upstream of said nicking enzyme site; a first nicking enzyme that

is capable of nicking at the nicking enzyme site of said forward template, and does not nick within said target sequence; a second nicking enzyme that is capable of nicking at the nicking enzyme site of said reverse template and does not nick within said target sequence; and a thermophilic polymerase under conditions wherein amplification is performed by multiple cycles of said polymerase extending said forward and reverse templates along said target sequence producing a double-stranded nicking enzyme site, and said nicking enzymes nicking at said nicking enzyme sites, producing an amplification product. In certain embodiments, the nicking enzyme sites on the forward and reverse templates are recognized by the same nicking enzyme, and only one nicking enzyme is used for the reaction.

In another embodiment, a method is provided for nucleic acid amplification comprising forming a mixture of a target nucleic acid comprising a single-stranded target sequence; a reverse template, wherein said reverse template comprises a nucleotide sequence comprising a recognition region at the 3’ end that is complementary to the 3’ end of the target sequence, a nicking enzyme site upstream of said recognition region, and a stabilizing region upstream of said nicking enzyme site; a first nicking enzyme that is capable of nicking at the nicking enzyme site of said reverse template, and does not nick within said target sequence; a thermophilic polymerase under conditions wherein said polymerase extends said reverse template along said target sequence; a forward template, wherein said forward template comprises a nucleic acid sequence comprising a recognition region at the 3’ end that is identical to the 5’end of the target sequence; and a second nicking enzyme that is capable of nicking at the nicking enzyme site of said forward template and does not nick within said target sequence; under conditions wherein amplification is performed by multiple cycles of said polymerase extending said forward and reverse templates along said target sequence producing a double-stranded nicking enzyme site, and said nicking enzymes nicking at said nicking enzyme sites, producing an amplification product. In certain embodiments, the nicking enzyme sites on the forward and reverse templates are recognized by the same nicking enzyme, and only one nicking enzyme is used for the reaction.

In other embodiments of the invention are provided methods for the separation of amplified nucleic acids obtained by the amplification methods of the invention. In yet further embodiments of the invention are provided methods for detecting and/or analyzing the amplified

nucleic acids obtained by the amplification methods of the invention, including, for example, methods using SYBR I, II, SYBR Gold, Pico Green, TOTO-3, and most intercalating dyes, molecular beacons, FRET, surface capture using immobilized probes with fluorescence, electrochemical, or colorimetric detection, mass spectrometry, capillary electrophoresis, the incorporation of labeled nucleotides to allow detection by capture or fluorescence polarization, lateral flow, and other methods involving capture probes. Methods using capture probes for detection include, for example, the use of a nucleic acid molecule (the capture probe) comprising a sequence that is complementary to the amplified product such that the capture probe binds to amplified nucleic acid. The reaction may, for example, further comprise an antibody directed against a molecule incorporated into or attached to the capture probe. Or, for example, the capture probe, or a molecule that binds to the capture probe, may incorporate, for example, an enzyme label, for example, peroxidase, alkaline phosphatase, or beta-galactosidase, a fluorescent label, such as, for example, fluorescein or rhodamine, or, for example, other molecules having chemiluminescent or bioluminescent activity. The embodiments of the present invention also comprise combinations of these detection and analysis methods.

Brief Description of the Drawings

Figures 1A-D are graphic drawings depicting mechanisms of the reactions of the present invention. Figure 1D is a legend for Figure 1.

Figure 2. 20% polyacrylamide gel of reaction products from a DNA NEAR assay.

The NEAR reaction was run for 2.5 minutes at 56°C, then heat denatured at 94 °C for 4 minutes. Six μL of the reaction was run on a 20% polyacrylamide gel at 160V for ~2.5 hrs. The gel was stained with SYBR II gel stain. Lane 1: NEAR reaction no target control for 25mer assay. Lane 2: NEAR reaction no target control for 27mer assay. Lane 3: NEAR reaction for 25mer assay with 3.5E+5 copies of genomic *Bacillus subtilis* DNA. Lane 4: NEAR reaction for 27mer assay with 1.1E+6 copies of genomic *Bacillus subtilis* DNA.

Figure 3. 20% polyacrylamide gel of reaction products from an RNA NEAR assay.

The NEAR reaction was run for 12 minutes at 56°C, then heat denatured at 94 °C for 4 minutes. Six μL of the reaction was run on a 20% polyacrylamide gel at 160V for -2.5 hrs. The gel was stained with SYBR II gel stain. Lane 1 & 2: NEAR reaction for 25mer assay with lE+6

copies of Ebola Armored RNA (Ambion). Lane 3 & 4: NEAR reaction no target control for 25mer assay. 25mer reaction products are outlined in the white box.

Figures 4A-B. Mass Spectrum of *Bacillus anthracis* DNA assay products.

A) 0 copies of target or B) 5E+5 copies of genomic DNA added to the NEAR reaction. The NEAR reaction was run for 10 minutes, then heat denatured at 94°C for 4 minutes. Ten micro liters of sample was injected into the LC/ESI-MS. The (-4) charge state of the 26mer product and its complementary sequence are outlined in a black box. The smaller adjacent peaks are the sodium adducts of the main product.

Figures 5A-C. Mass Spectrum of MS2 genomic RNA assay products.

A) 0 copies of target, B) lE+6 copies of MS2 genomic RNA, or C) lE+6 copies of synthetic target DNA added to the NEAR reaction. The NEAR reaction was run for 10 minutes, then heat denatured at 94°C for 4 minutes. Ten micro liters of sample was injected into the LC/ESI-MS. The (-4) charge state of the 27mer product and its complement sequence are outlined in a black box. The smaller adjacent peaks are the sodium adducts of the main product.

Figure 6. Real-time detection of NEAR assay amplification using intercalating fluorescent dyes.

Real-time amplification of *Yersinia pestis* genomic DNA at 500 copies (squares) compared to the no target control (NTC, open triangles). The reaction was run for 10 minutes at 58°C and monitored by the real-time fluorescence with SYBR II (n =5).

Figure 7. Real-time detection of NEAR assay amplification using fluorescence resonance energy transfer (FRET).

Real-time amplification of *Yersinia pestis* synthetic DNA at 10,000 copies (squares) compared to the no target control (NTC, open triangles). The reaction was run for 10 minutes at 57°C, n =3.

Figure 8. Francisella *tularensis* assay amplification detected in real-time using molecular beacons.

Either 0 copies (open triangles) or 1E+5 copies (squares) were added to the reaction mix and run for 10 minutes at 57.5 °C.

Figure 9. False alarm rate testing results comparing average *AVC* values.

Error bars denote one standard deviation. *Bacillus subtilis* NEAR assays were run for 10

min at 55°C in the presence and absence of *Bacillus subtilis* genomic DNA. Enzymes were heat denatured at 94°C for 4 min. A 10 µL sample was injected into the LC/ESI-MS and the area under the curve (AUC) of the product peaks were analyzed. True Positives contained 10,000 copies of *Bacillus subtilis* along with 990,000 copies of near neighbor *(Bacillus thuringiensis).* True Negatives contained 10,000 copies of *E. coli* with 990,000 copies of near neighbor, and water negatives contained no DNA as a control.

Figure 10. Replication study of the NEAR Assay using molecular beacon detection with different operators performing the experiments on two different days.

The NEAR reaction was run for 10 minutes at 57.5°C (in the presence and absence of 500 copies of *Francisella tularensis* genomic DNA) with a 4 min heat kill at 94°C. 300nM molecular beacon was added and monitored at 45, 50, and 57°C (n = 24).

Figure 11. Sensitivity of the NEAR reaction using molecular beacon detection.

The NEAR assay was run for 10 minutes 57.5°C. The reaction was stopped with a 4 min heat denaturation step at 94°C. 300nM molecular beacon was added and the fluorescence was monitored at 57.5°C (n =3). Fluorescence was monitored for beacon opening in the presence NEAR reactions amplified with 1E+6, 5E+5, 5E+4, 5E+2, 50, and 0 (NTC) input copies of *Francisella tularensis* genomic DNA, and compared to the background fluorescence of the beacon alone (MB).

Figure 12. Final concentration of amplified products in the NEAR reaction.

The NEAR reaction was run for 10 min at 55°C with varying copies of *Bacillus subtilis* genomic DNA. The reaction was stopped with a heat denaturation step at 94°C for 4 minutes. A 10 µL sample was injected into the LC/ESI-MS and the AUC of the product peak at 1944 Daltons was analyzed and compared to a standard curve.

Figure 13. Correlation of the input RNA target copy number to the final concentration of amplified products.

The Ebola NEAR assay was run for 12 min at 55°C with varying copies of synthetic RNA corresponding to the Ebola genome DNA. The reaction was stopped with a heat denaturation step at 94°C for 4 minutes. A 10 µL sample was injected into the LC/ESI-MS and the AUC of the product peak at 1936 Daltons was analyzed and compared to the standard curve of AUC values. (n =3)

Figure 14. Mass spec product analysis demonstrating NEAR reaction specificity.

The *Bacillus anthracis* NEAR reaction was run in the presence of a dilution of copies of Bacillus thuringiensis for 10 min at 56°C (n =3), then heat denatured at 94°C for 4 minutes. A 10 µL sample was injected into the LC/ESI-MS and AUC values of product peaks analyzed.

Figure 15. The effect of an interferent panel on the NEAR amplification.

Bacillus subtilis NEAR DNA reactions were run for 10 min at 55°C and heated to 94°C for 4 minutes to stop the reaction. Reactions were run in triplicate in the presence 1E+5 copies of *Bacillus subtilis* genomic DNA ("_1E+5") or with no target DNA present ("_0"). Sample x is the control assay with no interferent added. Interferents A through F were added at 50% reaction volume to the *Bacillus subtilis* assay. The *AUC* of mass spec product peaks were analyzed using a two-way ANOV A and Bonferroni t-test. (Key: A =none; B =House dust, skim milk; e =AZ test dust, humic acid; D =Diesel soot; E =Skim milk; F =Mold spores)

Figure 16. Gel electrophoresis results for the *Bacillus subtilis* / *Bacillus anthracis* DNA duplex reaction.

The NEAR reaction including templates for both *Bacillus subtilis (Bs)* and *Bacillus anthracis (Ba)* assays was run in the absence of target DNA (negative), in the presence of *Bacillus subtilis* only (positive for 27mer product), and in the presence of both *Bacillus subtilis* and *Bacillus anthracis* (positive for 27mer and 25mer product respectively). The target copy number used in this assay was 500,000 copies. The assay was run for 10 min at 57°C. Templates varied in concentration between the assays to control the amplification (100nM for *Bacillus anthracis* and 50 nM for *Bacillus subtilis).* Samples were run on a 20% polyacrylamide gel at 160 V for ~2 hours. The gel was stained with SYBR II fluorescent dye and imaged. The fluorescent bands were quantitated and analyzed as the integrated optical density (IOD) (n =8).

Figure 17. Specificity results for the *Bacillus subtilis* / *Bacillus anthracis* DNA duplex reaction shown by gel electrophoresis.

The NEAR reaction including templates for both a *Bacillus subtilis (Bs)* and *Bacillus anthracis (Ba)* assay was run in the absence of target DNA (negative), in the presence of *Bacillus subtilis* only (27mer product), and in the presence of both *Bacillus subtilis* and *Bacillus anthracis* (27mer and 25mer product respectively). The target copy number for each genome present in this assay was 500,000 copies. All reactions contained 500,000 copies of *Bacillus thuringiensis* as

clutter. Templates varied in concentration between the assays to control the amplification. The assay was run for 10 min at 57°C, heat denatured at 94°C for 4 min, and 6 μL was loaded on to a 20% gel run at 160 V for ~2 hours. The gel was stained with SYBR II fluorescent dye and imaged. The fluorescent bands were quantitated and analyzed as the integrated optical density (IOD).

Figure 18. Gel electrophoresis results for the MS2/Eboia RNA duplex reaction.

The NEAR reaction including templates for both a MS2 and Ebola assay was run in the absence of target RNA (negative, lanes 2-5), in the presence of MS2 only (27mer product, lanes 6 and 7), and in the presence of both MS2 and Ebola RNA (27mer and 25mer product respectively, lanes 8 and 9). The target copy number used in this assay was 1E+6 copies. The assay was run for 10 min at 57°C. Templates varied in concentration between the assays to control the amplification. Samples were run on a 20% polyacrylamide gel at 160 V for ~2.5 hours. The gel was stained with SYBR II fluorescent dye and imaged. The fluorescent bands were quantitated and analyzed as the integrated optical density (IOD).

Figure 19. Mass spec analysis of NEAR amplification of DNA from lysed spores.

Average AUC values from amplified product masses compared for lysed and unlysed samples. Lysed spore samples were then added to NEAR master mix and run for 10 minutes at 55°C, heat denatured for 4 minutes at 94°C, and run on the mass spec for analysis. AUC values of product peaks were averaged and compared (n =3).

Figure 20. Demonstration of the capture and extension approach for surface detection of the NEAR assay.

A.) Average binding (NEAR positive reaction product with no added polymerase), B.) 500,000 target (NEAR positive reaction product with added polymerase), and C.) No target (NEAR negative reaction with added polymerase) are compared. The NEAR assay was run for 10 minutes at 55°C, heat denatured at 94°C for 4 minutes, then added to the plate with capture probe bound to the surface on the 5' end. Polymerase is added to one well of the positive reaction. The plate is incubated at 55°C for 30 min, washed, SYBR II added, washed 3 times, and read on a Tecan plate reader (495 nm excitation/530 nm emission).

Figure 21. Pseudo-real-time fluorescence detection of the NEAR FRET assay with a single template immobilized on a surface in the presence (squares) and absence (open triangles)

of 1E+6 copies of genomic DNA.

NEAR reaction was performed in flat bottom 96-well plates covered with neutravidin. Solution of 1 μM FRET-labeled reverse template was incubated with gentle mixing for 1 hr at 37°C. Wells were washed 3 times with a PBS-Tween solution to release unbound template. NEAR reaction mix was added to the wells (one for each time point taken) and incubated at 58°C on a heating block in a shaking incubator set to 135 RPM. Time points were taken by adding 1 μL EDTA to the well to stop the reaction. The fluorescence was read from the bottom using a Tecan 100 plate reader.

Detailed Description

Provided herein are methods for the exponential amplification of short DNA or RNA sequences.

Target nucleic acids of the present invention include double-stranded and single-stranded nucleic acid molecules. The nucleic acid may be, for example, DNA or RNA. Where the target nucleic acid is an RNA molecule, the molecule may be, for example, double-stranded, single-stranded, or the RNA molecule may comprise a target sequence that is single-stranded. Target nucleic acids include, for example, genomic, plasmid, mitochondrial, cellular, and viral nucleic acid. The target nucleic acid may be, for example, genomic, chromosomal, plasmid DNA, a gene, any type of cellular RNA, or a synthetic oligonucleotide. By “genomic nucleic acid” is meant any nucleic acid from any genome, for example, including animal, plant, insect, and bacterial genomes, including, for example, genomes present in spores. Target nucleic acids further include microRNAs and siRNAs.

MicroRNAs, miRNAs, or small temporal RNAs (stRNAs), are short single-stranded RNA sequences, about 21-23 nucleotides long that are involved in gene regulation. MicroRNAs are thought to interfere with the translation of messenger RNAs as they are partially complementary to messenger RNAs. (see, for example, Ruvkun, GI, Science 294:797-99 (2001); Lagos-Quintana, M., et al., Science 294:854-58 (2001); Lau, N.C., et al, Science 294:858-62 (2001); Lee, R.C., and Ambros, V., Science 294:862-64 (2001); Baulcombe, D., et al., Science 297:2002-03 (2002); Llave, c., Science 297:2053-56 (2002); Hutvagner, G., and Zamore, P.D., Science 297:2056-60 (2002)). MicroRNA may also have a role in the immune system, based on

studies recently reported in knock-out mice. (see, for example, Wade, N., "Studies Reveal and Immune System Regulator" New York Times, April 27, 2007). MicroRNA precursors that may also be detected using the methods of the present invention include, for example, the primary transcript (pri-miRNA) and the pre-miRNA stem-loop-structured RNA that is further processed into miRNA.

Short interfering RNAs, or siRNAs are at least partially double-stranded, about 20-25 nucleotide long RNA molecules that are found to be involved in RNA interference, for example, in the down-regulation of viral replication or gene expression (see for example Zamore et al., 2000, Cell, 101,25-33; Bass, 2001, Nature, 411, 428-429; Elbashir et al., 2001, Nature, 411, 494-498; and Kreutzer et al., International PCT Publication No. WO 00/44895; Zernicka-Goetz et al., International PCT Publication No. WO 01/36646; Fire, International PCT Publication No. WO 99/32619; Plaetinck et al., International PCT Publication No. WO 00101846; Mello and Fire, International PCT Publication No. WO 01/29058; Deschamps-Depaillette, International PCT Publication No. WO 99107409; and Li et al., International PCT Publication No. WO 00/44914; Allshire, 2002, Science, 297,1818-1819; Volpe et al., 2002, Science, 297,1833-1837; Jenuwein, 2002, Science, 297, 2215-2218; and Hall et al., 2002, Science, 297, 2232-2237; Hutvagner and Zamore, 2002, Science, 297, 2056-60; McManus et al., 2002, RNA, 8, 842-850; Reinhart et al., 2002, Gene & Dev., 16, 1616-1626; and Reinhart & Bartel, 2002, Science, 297, 1831).

The use of the term "target sequence" may refer to either the sense or antisense strand of the sequence, and also refers to the sequences as they exist on target nucleic acids, amplified copies, or amplification products, of the original target sequence. The amplification product may be a larger molecule that comprises the target sequence, as well as at least one other sequence, or other nucleotides. The length of the target sequence, and the guanosine:cytosine (GC) concentration (percent), is dependent on the temperature at which the reaction is run; this temperature is dependent on the stability of the polymerases and nicking enzymes used in the reaction. Those of ordinary skill in the art may run sample assays to determine the appropriate length and GC concentration for the reaction conditions. For example, where the polymerase and nicking enzyme are stable up to 60°C, then the target sequence may be, for example, from 19 to 50 nucleotides in length, or for example, from 20 to 45, 20 to 40, 22-35, or 23 to 32 nucleotides in length. The GC concentration under these conditions may be, for example, less than 60%, less

than 55%, less than 50%, or less than 45%. The target sequence should not contain nicking sites for any nicking enzymes that will be included in the reaction mix.

The target sequences may be amplified in many types of samples including, but not limited to samples containing spores, viruses, cells, nucleic acid from prokaryotes or eukaryotes, or any free nucleic acid. For example, the assay can detect the DNA on the outside of spores without the need for lysis. The sample may be isolated from any material suspected of containing the target sequence. For example, for animals, for example, mammals, such as, for example, humans, the sample may comprise blood, bone marrow, mucus, lymph, hard tissues, for example, liver, spleen, kidney, lung, or ovary, biopsies, sputum, saliva, tears, feces, or urine. Or, the target sequence may be present in air, plant, soil, or other materials suspected of containing biological organisms.

Target sequences may be present in samples that may also contain environmental and contaminants such as dust, pollen, and diesel exhaust, or clinically relevant matrices such as urine, mucus, or saliva. Target sequences may also be present in waste water, drinking water, air, milk, or other food. Depending on the concentration of these contaminants, sample purification methods known to those of ordinary skill in the art may be required to remove inhibitors for successful amplification. Purification may, for example, involve the use of detergent lysates, sonication, vortexing with glass beads, or a French press. This purification could also result in concentration of the sample target. Samples may also, for be further purified, for example, by filtration, phenol extraction, chromatography, ion exchange, gel electrophoresis, or density dependent centrifugation. The sample can be added directly to the reaction mix or pre-diluted and then added.

An oligonucleotide is a molecule comprising two or more deoxyribonucleotides or ribonucleotides, for example, more than three. The length of an oligonucleotide will depend on how it is to be used. The oligonucleotide may be derived synthetically or by cloning.

The term "complementary" as it refers to two nucleic acid sequences generally refers to the ability of the two sequences to form sufficient hydrogen bonding between the two nucleic acids to stabilize a double-stranded nucleotide sequence formed by hybridization of the two nucleic acids.

As used herein, "hybridization" and "binding" are used interchangeably and refer to the

non-covalent binding or "base pairing" of complementary nucleic acid sequences to one another. Whether or not a particular probe remains base paired with a polynucleotide sequence depends on the degree of complementarity, the length of the probe, and the stringency of the binding conditions. The higher the stringency, the higher must be the degree of complementarity, and/or the longer the probe for binding or base pairing to remain stable.

As used herein, "stringency" refers to the combination of conditions to which nucleic acids are subjected that cause double-stranded nucleic acid to dissociate into component single strands such as pH extremes, high temperature, and salt concentration. The phrase "high stringency" refers to hybridization conditions that are sufficiently stringent or restrictive such that only specific base pairings will occur. The specificity should be sufficient to allow for the detection of unique sequences using an oligonucleotide probe or closely related sequence under standard Southern hybridization protocols (as described in J. Mol. Biol. 98:503 (1975)).

Templates are defined as oligonucleotides that bind to a recognition region of the target and also contain a nicking enzyme binding region upstream of the recognition region and a stabilizing region upstream to the nicking enzyme binding region.

By "recognition region" is meant a nucleic acid sequence on the template that is complementary to a nucleic acid sequence on the target sequence. By "recognition region on the target sequence" is meant the nucleotide sequence on the target sequence that is complementary to, and binds to, the template.

By "stabilizing region" is meant a nucleic acid sequence having, for example, about 50% GC content, designed to stabilize the molecule for, for example, the nicking and/or extension reactions.

In describing the positioning of certain sequences on nucleic acid molecules, such as, for example, in the target sequence, or the template, it is understood by those of ordinary skill in the art that the terms "3'" and "5'" refer to a location of a particular sequence or region in relation to another. Thus, when a sequence or a region is 3' to or 3' of another sequence or region, the location is between that sequence or region and the 3' hydroxyl of that strand of nucleic acid. When a location in a nucleic acid is 5' to or 5' of another sequence or region, that means that the location is between that sequence or region and the 5' phosphate of that strand of nucleic acid.

The polymerase is a protein able to catalyze the specific incorporation of nucleotides to

extend a 3' hydroxyl terminus of a primer molecule, such as, for example, the template oligonucleotide, against a nucleic acid target sequence. The polymerase may be, for example, thermophilic so that it is active at an elevated reaction temperature. It may also, for example, have strand displacement capabilities. It does not, however, need to be very processive (30-40 nucleotides for a single synthesis is sufficient). If the polymerase also has reverse transcription capabilities (such as Bst (large fragment), 9°N, Therminator, Therminator II, etc.) the reaction can also amplify RNA targets in a single step without the use of a separate reverse transcriptase. More than one polymerase may be included in the reaction, in one example one of the polymerases may have reverse transcriptase activity and the other polymerase may lack reverse transcriptase activity. The polymerase may be selected from, for example, the group consisting of one or more of the polymerases listed in Table 1.

Table 1

Polymerase
Bst DNA polymerase
Bst DNA polymerase (Large fragment)
9°Nm DNA polymerase
Phi29 DNA polymerase
DNA polymerase I *(E.coli)*
DNA polymerase I, Large (Klenow) fragment
Klenow fragment (3'-5' exo-)
T4 DNA polymerase
T7 DNA polymerase
Deep VentR™ (exo-) DNA Polymerase
Deep VentR™DNA Polymerase
DyNAzyme™ EXT DNA
DyNAzyme™ II Hot Start DNA Polymerase
Phusion™ High-Fidelity DNA Polymerase
Therminator™ DNA Polymerase
Therminator™ II DNA Polymerase
VentR®DNA Polymerase
VentR® (exo-) DNA Polymerase
RepliPHFM Phi29 DNA Polymerase
rBst DNA Polymerase, Large Fragment (IsoTherm™ DNA Polymerase)
MasterAmpTM AmpliThermTM DNA Polymerase
Tag DNA polymerase
Tth DNA polymerase
Tfl DNA polymerase
Tgo DNA polymerase
SP6 DNA polymerase
Tbr DNA polymerase
DNA polymerase Beta
ThermoPhi DNA polymerase

"Nicking" refers to the cleavage of only one strand of the double-stranded portion of a fully or partially double-stranded nucleic acid. The position where the nucleic acid is nicked is referred to as the nicking site or nicking enzyme site. The recognition sequence that the nicking enzyme recognizes is referred to as the nicking enzyme binding site. "Capable of nicking" refers to an enzymatic capability of a nicking enzyme.

The nicking enzyme is a protein that binds to double-stranded DNA and cleaves one strand of a double-stranded duplex. The nicking enzyme may cleave either upstream or downstream of the binding site, or nicking enzyme recognition site. In exemplary embodiments, the reaction comprises the use of nicking enzymes that cleave or nick downstream of the binding site (top strand nicking enzymes) so that the product sequence does not contain the nicking site. Using an enzyme that cleaves downstream of the binding site allows the polymerase to more easily extend without having to displace the nicking enzyme. The nicking enzyme must be functional in the same reaction conditions as the polymerase, so optimization between the two ideal conditions for both is necessary. Nicking enzymes are available from, for example, New England Biolabs (NEB) and Fermentas. The nicking enzyme may, for example, be selected from the group consisting of one or more of the nicking enzymes listed in Table 2.

Table 2

Nicking Enzyme	**Alternate Name**
Nb.BbvCI	
Nb.Bpu101	
Nb.Bsal	
Nb.Bsml	
Nb.BsrDI	
Nb.BstNBIP	
Nb.BstSEIP	
Nb.Btsl	
Nb.Sapl	
Nt.Alwl	
Nt.BbvCI	
Nt.BhaIIiP	
Nt.Bpu101	
Nt.Bpu10IB	
Nt.Bsal	
Nt.BsmAI	
Nt.BsmBI	
Nt.BspD61	
Nt.BspQI	
Nt.Bst91	
Nt.BstNBI	N.BstNB I
Nt.BstSEI	
Nt.CviARORFMP	
Nt.CviFRORFAP	
Nt.CviPIl	Nt.CviPlim
Nt.CviQII	
Nt.CviQXI	
Nt.EsaSS1198P	
Nt.Mlyl	
Nt.Sapl	

Nicking enzymes may be, for example, selected from the group consisting of Nt.BspQI(NEB), Nb.BbvCI(NEB), Nb.BsmI(NEB), Nb.BsrDI(NEB), Nb.BtsI(NEB), Nt.AlwI(NEB), Nt.BbvCI(NEB), Nt.BstNBI(NEB), Nt.CviPII(NEB), Nb.BpulOl(Fermantas), and Nt.BpulOl(Fermentas). In certain embodiments, the nicking enzyme is selected from the group consisting of Nt.NBst.NBI, Nb.BsmI, and Nb.BsrDI. Those of ordinary skill in the art are

aware that various nicking enzymes other than those mentioned specifically herein may be used in the methods of the present invention.

Nicking enzymes and polymerases of the present invention may be, for example, stable at room temperature, the enzymes may also, for example, be stable at temperatures up to 37°C, 42°C, 60°C, 65°C, 70°C, 75°C, 80°C, or 85°C. In certain embodiments, the enzymes are stable up to 60°C.

Product or amplified product is defined as the end result of the extension of the template along the target that is nicked, released, and then feeds back into the amplification cycle as a target for the opposite template.

A “native nucleotide” refers to adenylic acid, guanylic acid, cytidylic acid, thymidylic acid, or uridylic acid. A “derivatized nucleotide” is a nucleotide other than a native nucleotide.

The reaction may be conducted in the presence of native nucleotides, such as, for example, dideoxyribonucleoside triphosphates (dNTPs). The reaction may also be carried out in the presence of labeled dNTPs, such as, for example, radiolabels such as, for example, ^{32}p, ^{33}p, ^{125}1, or ^{35}S, enzyme labels such as alkaline phosphatase, fluorescent labels such as fluorescein isothiocyanate (FITC), biotin, avidin, digoxigenin, antigens, haptens, or fluorochromes. These derivatized nucleotides may, for example, be present in the templates.

By “constant temperature,” “isothermal conditions” or “isothermally” is meant a set of reaction conditions where the temperature of the reaction is kept essentially constant during the course of the amplification reaction. An advantage of the amplification method of the present invention is that the temperature does not need to be cycled between an upper temperature and a lower temperature. The nicking and the extension reaction will work at the same temperature or within the same narrow temperature range. However, it is not necessary that the temperature be maintained at precisely one temperature. If the equipment used to maintain an elevated temperature allows the temperature of the reaction mixture to vary by a few degrees, this is not detrimental to the amplification reaction, and may still be considered to be an isothermal reaction.

The term “multiplex amplification” refers to the amplification of more than one nucleic acid of interest. For example, it can refer to the amplification of multiple sequences from the same sample or the amplification of one of several sequences in a sample as discussed, for

example, in U.S. Patent Nos. 5,422,252; and 5,470,723, which provide examples of multiplex strand displacement amplification. The term also refers to the amplification of one or more sequences present in multiple samples either simultaneously or in step-wise fashion.

Template Design

Forward and Reverse templates are designed so that there is a stabilizing region at the 5' end, a nicking site downstream of the stabilizing region, and a recognition region downstream of the nicking site on the 3' end of the oligonucleotide. The total oligo length can range from 19 to 40, for example from 19-40, 23-40, 20-24, 23-24, 23-32, 25-40, 27-40, or 27-35 nucleotides depending on the length of each individual region, the temperature, the length of the target sequence, and the GC concentration. The templates may be designed so that they, together, would bind to less than or equal to 100% of the target sequence, one binding to the sense strand, and one to the antisense strand. For example, where the forward template binds to about 60% of the target antisense strand, the reverse template may, for example, bind to about 40% of the target sense strand. The templates may be designed to allow for spacer bases on the target sequence, that do not bind to either template. The templates thus may be designed to bind to about 30%, about 40%, about 50%, or about 60% of the target sequence.

The recognition region of the forward template is designed to be identical to the 5' region of the target sense strand and complementary to the 3' end of the target site antisense strand, for example, 8-16, 9-16, 10-16, 10-15, or 11-14 nucleotides long. In exemplary embodiments, the length is 12-13 nucleotides. The recognition region of the reverse template is designed to be complementary to the 3' end of the target site sense strand, for example, 8-16, 9-16, 10-16, 1015, or 11-14 nucleotides long. In exemplary embodiments, the length is 12-13 nucleotides.

In certain embodiments, the lengths of the recognition regions are adjusted so that there is at least one nucleotide in the target sequence that is not in the forward template's recognition region and also does not have its complement in the reverse template's recognition region. These spacer bases are nucleotides contained within the target sequence that lie in between the 3' ends of the forward and reverse templates. In certain embodiments, 5 spacer bases or less are present in the target sequence. In exemplary embodiments, the number of spacer bases is 2 to 3. In certain embodiments, the number of spacer bases is 1, 2, 3, 4, or 5. These spacer bases allow for

distinction of the true amplified product from any background products amplified by extension due to overlapping templates in a similar manner to primer-dimers. This consideration allows for improved discrimination between background and amplification of true target. However, these spacer bases are not required for the amplification to proceed.

The nicking site sequence of the template depends on which nicking enzyme is chosen for each template. Different nicking enzymes may be used in a single assay, but a simple amplification may, for example, employ a single nicking enzyme for use with both templates. Thus, the embodiments of the present invention include those where both templates comprise recognition sites for the same nicking enzyme, and only one nicking enzyme is used in the reaction. In these embodiments, both the first and second nicking enzymes are the same. The present invention also includes those embodiments where each template comprises a recognition site for a different nicking enzyme, and two nicking enzymes are used in the reaction.

For example, in the case of Nt.BstNBI, the enzyme binding site is 5'-GAGTC-3' and the enzyme nicks the top strand four nucleotides down stream of this site (i.e., GAGTCNNNN^). The amplification reaction shows little dependence on the sequence of these four nucleotides (N), though optimal sequence of this region is 25% or less GC content and with a thymine adjacent to the 5' nucleotide of the binding region. The latter stipulation allows for the priming ability of products that have an additional adenine added on by the polymerase. The sequence of the four nucleotides can be optimized to create or eliminate the presence of hairpins, self-dimers, or heterodimers, depending on the application.

The stabilizing region on the 5' end of the template oligonucleotide is designed to be roughly 50% GC. Thus, the GC content may be, for example, about 40%-60%, about 42%-58%, about 44%-56%, about 46%-54%, about 48%-52%, or about 49%-51 %. These parameters result in a stabilizing region length of 8-11 nucleotides for the Nt.BstNBI enzyme, though lengths as short as 6 and as long as 15 nucleotides have been tested and were shown to work in this amplification method. Longer stabilizing regions or increased %GC to greater than 50% could further stabilize the nicking and extension reactions at higher reaction temperatures. The sequence of the 5' stabilizing regions of forward and reverse templates are usually identical, but can be varied if the aim is to capture each product strand independently. The sequence of this region should not interfere with the nicking site or the recognition region, though short internal

hairpins within the template sequence have been shown to have improved real-time results.

The templates of the present invention may include, for example, spacers, blocking groups, and modified nucleotides. Modified nucleotides are nucleotides or nucleotide triphosphates that differ in composition and/or structure from natural nucleotide and nucleotide triphosphates. Modified nucleotide or nucleotide triphosphates used herein may, for example, be modified in such a way that, when the modifications are present on one strand of a double-stranded nucleic acid where there is a restriction endonuclease recognition site, the modified nucleotide or nucleotide triphosphates protect the modified strand against cleavage by restriction enzymes. Thus, the presence of the modified nucleotides or nucleotide triphosphates encourages the nicking rather than the cleavage of the double-stranded nucleic acid. Blocking groups are chemical moieties that can be added to the template to inhibit target sequence-independent nucleic acid polymerization by the polymerase. Blocking groups are usually located at the 3' end of the template. Examples of blocking groups include, for example, alkyl groups, non-nucleotide linkers, phosphorothioate, alkane-diol residues, peptide nucleic acid, and nucleotide derivatives lacking a 3'-OH, including, for example, cordycepin. Examples of spacers, include, for example, C3 spacers. Spacers may be used, for example, within the template, and also, for example, at the 5' end, to attach other groups, such as, for example, labels.

Detailed Mechanism of Amplification

NEAR amplification requires the presence of a nucleic acid target, at least two template oligonucleotides, a thermophilic nicking enzyme, a thermophilic polymerase, and buffer components all held at the reaction temperature. The recognition region of the templates interacts with the complementary target sequence. Since the melting temperature of the complementary regions of the target and template is well below the reaction temperature, the interaction between the two nucleic acid strands is transient, but allows enough time for a thermophilic polymerase to extend from the 3' end of the template along the target strand. Experiments have shown that certain polymerases bind to single-stranded oligonucleotides. The pre-formation of this complex can facilitate the speed of the amplification process.

For a double-stranded target, both templates can interact with the corresponding target strands simultaneously (forward template with the antisense strand and reverse template with the

sense strand) during the normal breathing of double-stranded DNA. The target may also be generated by a single or double nick sites within the genome sequence. For a single-stranded target (either RNA or DNA), the reverse template binds and extends first (Figure 1, Step 1 and 2). The extended sequence contains the complement to the forward template. The forward template then displaces a region of the target and binds to the 3' synthesized region complementary to the recognition region of the forward template (Step 3). Alternatively, another reverse template can also displace the initial extended reverse template at the recognition region to create a single-stranded extended reverse template for the forward template to bind. The initial binding and extension of the templates is facilitated by a non-processive polymerase that extends shorter strands of DNA so that the melting temperature of the synthesized product is at or near the reaction temperature; therefore, a percentage of the product becomes single-stranded once the polymerase dissociates. The single-stranded product is then available for the next template recognition site to bind and polymerase to extend.

The forward template is extended to the *5'* end of the reverse template, creating a double-stranded nicking enzyme binding site for the reverse template (Step 5). The nicking enzyme then binds to the duplex and nicks directly upstream of the recognition sequence of the reverse template strand (in the case of a top-strand nicking enzyme) (Step 6). The nucleic acid sequence downstream of the nick is either released (if the melting temperature is near the reaction temperature) and/or is displaced by the polymerase synthesis from the 3-OH nick site.

Polymerase extends along the forward template to the 5' end of the forward template (Step 8).

The double-strand formed from the extension of both templates creates a nicking enzyme binding site on either end of the duplex. This double-strand is termed the NEAR amplification duplex. When nicking enzyme binds and nicks, either the target product located in between the two nick sites (with 5'-phosphate and 3-OH) is released, usually ranging in length from (but is not limited to) 23 to 29 bases (Steps 9-11A), or the singly-nicked product containing the target product and the reverse complement of the nick site and stability region of the template (usually 36 to 48 bases in length) is released (Steps 9-11B). The ratio of products 1 to 2 can be adjusted by varying the concentrations of the templates. The forward:reverse template ratio may vary from, for example, molar ratios of 100:1, 75:1; 50:1, 40:1, 30:1, 20:1, 10:1, 5:1, 2.5:1, 1:1, 1:2.5,

1:5, 1:10, 1:20, 1:30, 1:40, 1:50, 1:75, or 1:100. The ratio of products (A to B) is dependent on the ratio of nicking enzyme to polymerase, *i.e.* a higher concentration of polymerase results in more of the longer length product (B) since there is comparatively less nicking enzyme to nick both strands simultaneously before the polymerase extends. Since displaced/released product of the reverse template feeds into the forward template and vice versa, exponential amplification is achieved. The nicking enzyme:polymerase ratio may vary from, for example, enzyme unit ratios of 20:1, 15:1; 10:1, 5:1, 4:1, 3:1, 2:1, 1.5:1, 1:1, 1:1.5, 1:2, 1:3, 1:4, 1:5, 1:10, 1:15, 1:20. In certain embodiments, the ratio of nicking enzyme to polymerase may, for example, be 1:3, 1:2, 1:1.5, or 1:0.8. Those of ordinary skill in the art recognize that these ratios may represent rounded values. This nicking and polymerase extension process continues until one of the resources (usually dNTPs or enzyme) is exhausted.

The time that the reaction is run may vary from, for example, 1-20 minutes, or 1-10, 1-8 , 1-5, 1-2.5, 2.5-5, 2.5-8, 2.5-10, or 2.5-20 minutes.

The methods of the present invention do not require the use of temperature cycling, as often is required in methods of amplification to dissociate the target sequence from the amplified nucleic acid. The temperature of the reaction may vary based on the length of the sequence, and the GC concentration, but, as understood by those of ordinary skill in the art, the temperature should be high enough to minimize non-specific binding. The temperature should also be suitable for the enzymes of the reaction, the nicking enzyme and the polymerase. For example, the reaction may be run at 37°C-85°C, 37°C-60°C, 54°C-60°C, and, in exemplary embodiments, from 55°C-59°C.

The polymerase may be mixed with the target nucleic acid molecule before, after, or at 15 the same time as, the nicking enzyme. In exemplary embodiments, a reaction buffer is optimized to be suitable for both the nicking enzyme and the polymerase.

Reactions may be allowed to completion, that is, when one of the resources is exhausted. Or, the reaction may be stopped using methods known to those of ordinary skill in the art, such as, for example, heat denaturation, or the addition of EDTA, high salts, or detergents. In exemplary embodiments, where mass spectrometry is to be used following amplification, EDTA may be used to stop the reaction.

Reaction Components

In a 1.5 mL Eppendorf tube combine the following reagents in order from top to bottom:

Reagent Added:	µL Per Reaction
H_2O	31.4
10X Thermopol Buffer (NEB)	5
10X NEB Buffer 3	2.5
100 mM $MgSO_4$	4.5
10 mM dNTPs	1.5
8 U/µl Bst Pol	0.6
10 U/µl N.BstNBI	1.5
20 µM Forward Template	0.25
20 µM Reverse Template	0.25
Total reaction mixture	47.5
Target sample	2.5
Total Reaction Volume	50 µL

The concentrations of components for the reaction conditions in this example are as follows:

Concentration	**Component**
45.7mM	Tris-HGI
13.9 mM	KCl
10 mM	$(NH_4)_2SO_2$
50 mM	NaCl
0.5mM	DTT
15 mM	$MgCl_2$
0.10%	Triton X-100
0.008 mM	EDTA
6 µg/mL	BSA
3.90%	Glycerol (can be lower if using more concentrated enzyme stock)
0.3 U/µL	NT.BstNBI
0.1-0.4 U/µL	Bst polymerase (large fragment)
0.1 µM	Forward template
0.1 µM	Reverse template

Variations in buffer conditions, $MgSO_4$ concentration, polymerase concentration, and template concentrations all can be optimized based on the assay sequence and desired detection

method. The amount of glycerol may, for example, be lowered if a more concentrated enzyme stock is used. Also, those of ordinary skill in the art recognize that the reaction may be run without EDTA or BSA; these components may be present in the reaction as part of the storage buffers for the enzymes. The volumes can be scaled for larger or smaller total reaction volumes. The volume is usually between 5 µL and 100 µL.

The template concentrations are typically in excess of the concentration of target. The concentrations of the forward and reverse templates can be at the same or at different concentrations to bias the amplification of one product over the other. The concentration of each is usually between 10 nM and 1µM.

Additives such as BSA, non-ionic detergents such as Triton X-100 or Tween-20, DMSO, DTT, and RNase inhibitor may be included for optimization purposes without adversely affecting the amplification reaction.

Preparing/Adding Target

Targets may be diluted in 1 x Thermopol Buffer II, 1 x TE (pH 7.5) or H_20. Hot start conditions allow for faster, more specific amplification. In this case, the reaction mix (minus either enzymes or templates and target) is heated to the reaction temperature for 2 minutes, after which the reaction mix is added to the other component (enzymes or templates/target). The target can be added in any volume up to the total amount of water required in the reaction. In this case, the target would be diluted in water. In the example above for a 50 µL total reaction volume, 2.5 µL of the prepared target should be added per reaction to bring the total reaction volume to 50 µL.

Running the Reaction

The reaction is run at a constant temperature, usually between 54°C and 60°C for the enzyme combination of Bst polymerase (large fragment) and Nt.Bst.NB 1 nicking enzyme. Other enzyme combinations may be used and the optimal reaction temperature will be based on the optimal temperature for both the nicking enzyme and polymerase to work in concert as well as the melting temperature of the reaction products. The reaction is held at temperature for 2.5 to 10 minutes until the desired amount of amplification is achieved. The reaction may be stopped by

either a heat denaturation step to denature the enzymes (when using enzymes that can be heat-killed). Alternatively, the reaction may be stopped by adding EDTA to the reaction.

Readout

The amplified target sequence may be detected by any method known to one of ordinary skill in the art. By way of non-limiting example, several of these known methods are presented herein. In one method, amplified products may be detected by gel electrophoresis, thus detecting reaction products having a specific length. The nucleotides may, for example, be labeled, such as, for example, with biotin. Biotin-labeled amplified sequences may be captured using avidin bound to a signal generating enzyme, for example, peroxidase.

Nucleic acid detection methods may employ the use of dyes that specifically stain double-stranded DNA. Intercalating dyes that exhibit enhanced fluorescence upon binding to DNA or RNA are a basic tool in molecular and cell biology. Dyes may be, for example, DNA or RNA intercalating fluorophores and may include but are not limited to the following examples: Acridine orange, ethidium bromide, Hoechst dyes, PicoGreen, propidium iodide, SYBR I (an asymmetrical cyanine dye), SYBR II, TOTO (a thiaxole orange dimer) and YOYO (an oxazole yellow dimer). Dyes provide an opportunity for increasing the sensitivity of nucleic acid detection when used in conjunction with various detection methods and may have varying optimal usage parameters. For example ethidium bromide is commonly used to stain DNA in agarose gels after gel electrophoresis and during peR (Hiquchi et al., Nature Biotechnology 10; 413-417, April 1992), propidium iodide and Hoechst 33258 are used in flow cytometry to determine DNA ploidy of cells, SYBR Green 1 has been used in the analysis of double-stranded DNA by capillary electrophoresis with laser induced fluorescence detection and Pico Green has been used to enhance the detection of double-stranded DNA after matched ion pair polynucleotide chromatography (Singer et al., Analytical Biochemistry 249,229-238 1997).

Nucleic acid detection methods may also employ the use of labeled nucleotides incorporated directly into the target sequence or into probes containing complementary sequences to the target of interested. Such labels may be radioactive and/or fluorescent in nature and can be resolved in any of the manners discussed herein.

Methods of detecting and/or continuously monitoring the amplification of nucleic acid

products are also well known to those skilled in the art and several examples are described below.

The production or presence of target nucleic acids and nucleic acid sequences may be detected and monitored by Molecular Beacons. Molecular Beacons are hair-pin shaped oligonucleotides containing a fluorophore on one end and a quenching dye on the opposite end. The loop of the hair-pin contains a probe sequence that is complementary to a target sequence and the stem is formed by annealing of complementary arm sequences located on either side of the probe sequence. A fluorophore and a quenching molecule are covalently linked at opposite ends of each arm. Under conditions that prevent the oligonucleotides from hybridizing to its complementary target or when the molecular beacon is free in solution the fluorescent and quenching molecules are proximal to one another preventing fluorescence resonance energy transfer (FRET). When the molecular beacon encounters a target molecule, hybridization occurs; the loop structure is converted to a stable more rigid conformation causing separation of the fluorophore and quencher molecules leading to fluorescence (Tyagi et al. Nature Biotechnology 14: March 1996,303-308). Due to the specificity of the probe, the generation of fluorescence is exclusively due to the synthesis of the intended amplified product.

Molecular beacons are extraordinarily specific and can discern a single nucleotide polymorphism. Molecular beacons can also be synthesized with different colored fluorophores and different target sequences, enabling several products in the same reaction to be quantitated simultaneously. For quantitative amplification processes, molecular beacons can specifically bind to the amplified target following each cycle of amplification, and because non-hybridized molecular beacons are dark, it is not necessary to isolate the probe-target hybrids to quantitatively determine the amount of amplified product. The resulting signal is proportional to the amount of amplified product. This can be done in real time. As with other real time formats, the specific reaction conditions must be optimized for each primer/probe set to ensure accuracy and precision.

The production or presence of target nucleic acids and nucleic acid sequences may also be detected and monitored by Fluorescence resonance energy transfer (FRET). FRET is an energy transfer mechanism between two chromophores: a donor and an acceptor molecule. Briefly, a donor fluorophore molecule is excited at a specific excitation wavelength. The

subsequent emission from the donor molecule as it returns to its ground state may transfer excitation energy to the acceptor molecule through a long range dipole-dipole interaction. The intensity of the emission of the acceptor molecule can be monitored and is a function of the distance between the donor and the acceptor, the overlap of the donor emission spectrum and the acceptor absorption spectrum and the orientation of the donor emission dipole moment and the acceptor absorption dipole moment. FRET is a useful tool to quantify molecular dynamics, for example, in DNA-DNA interactions as seen with Molecular Beacons. For monitoring the production of a specific product a probe can be labeled with a donor molecule on one end and an acceptor molecule on the other. Probe-target hybridization brings a change in the distance or orientation of the donor and acceptor and FRET change is observed. (Joseph R. Lakowicz, "Principles of Fluorescence Spectroscopy", Plenum Publishing Corporation, 2nd edition (July 1, 1999)).

The production or presence of target nucleic acids and nucleic acid sequences may also be detected and monitored by Mass Spectrometry. Mass Spectrometry is an analytical technique that may be used to determine the structure and quantity of the target nucleic acid species and can be used to provide rapid analysis of complex mixtures. Following the method, samples are ionized, the resulting ions separated in electric and/or magnetic fields according to their mass-to-charge ratio, and a detector measures the mass-to-charge ratio of ions. (Crain, P. F. and McCloskey, J. A., Current Opinion in Biotechnology 9: 25-34 (1998)). Mass spectrometry methods include, for example, MALDI, MALDIITOF, or Electrospray. These methods may be combined with gas chromatography (GC/MS) and liquid chromatography (LC/MS). MS has been applied to the sequence determination of DNA and RNA oligonucleotides (Limbach P., MassSpectrom. Rev. 15: 297-336 (1996); Murray K., J. Mass Spectrom. 31: 1203-1215 (1996)). MS and more particularly, matrix-assisted laser desorption/ionization MS (MALDI MS) has the potential of very high throughput due to high-speed signal acquisition and automated analysis off solid surfaces. It has been pointed out that MS, in addition to saving time, measures an intrinsic property of the molecules, and therefore yields a significantly more informative signal (Koster H. et al., Nature Biotechnol., 14: 1123-1128 (1996)).

The production or presence of target nucleic acids and nucleic acid sequences may also be detected and monitored by various methods of gel electrophoresis. Gel electrophoresis

involves the separation of nucleic acids through a matrix, generally a cross-linked polymer, using an electromotive force that pulls the molecules through the matrix. Molecules move through the matrix at different rates causing a separation between products that can be visualized and interpreted via anyone of a number of methods including but not limited to; autoradiography, phosphorimaging, and staining with nucleic acid chelating dyes.

The production or presence of target nucleic acids and nucleic acid sequences may also be detected and monitored by capillary gel electrophoresis. Capillary-gel Electrophoresis (CGE) is a combination of traditional gel electrophoresis and liquid chromatography that employs a medium such as polyacrylamide in a narrow bore capillary to generate fast, high-efficient separations of nucleic acid molecules with up to single base resolution. CGE is commonly combined with laser induced fluorescence (LIF) detection where as few as six molecules of stained DNA can be detected. CGE/LIF detection generally involves the use of fluorescent DNA intercalating dyes including ethidium bromide, YOYO and SYBR Green 1 but can also involve the use of fluorescent DNA derivatives where the fluorescent dye is covalently bound to the DNA. Simultaneous identification of several different target sequences can be made using this method.

The production or presence of target nucleic acids and nucleic acid sequences may also be detected and monitored by various surface capture methods. This is accomplished by the immobilization of specific oligonucleotides to a surface producing a biosensor that is both highly sensitive and selective. Surfaces used in this method may include but are not limited to gold and carbon and may use a number of covalent or noncovalent coupling methods to attach the probe to the surface. The subsequent detection of a target DNA can be monitored by a variety of methods.

Electrochemical methods generally involve measuring the cathodic peak of intercalators, such as methylene blue, on the DNA probe electrode and visualized with square wave voltammograms. Binding of the target sequence can be observed by a decrease in the magnitude of the voltammetric reduction signals of methylene blue as it interacts with dsDNA and ssDNA differently reflecting the extent of the hybrid formation.

Surface Plasmon Resonance (SPR) can also be used to monitor the kinetics of probe attachment as well as the process of target capture. SPR does not require the use of fluorescence probes or other labels. SPR relies on the principle of light being reflected and refracted on an

interface of two transparent media of different refractive indexes. Using monochromatic and p-polarized light and two transparent media with an interface comprising a thin layer of gold, total reflection of light is observed beyond a critical angle, however the electromagnetic field component of the light penetrates into the medium of lower refractive index creating an evanescent wave and a sharp shadow (surface plasmon resonance). This is due to the resonance energy transfer between the wave and the surface plasmons. The resonance conditions are influenced by the material absorbed on the thin metal film and nucleic acid molecules, proteins and sugars concentrations are able to be measured based on the relation between resonance units and mass concentration.

The production or presence of target nucleic acids and nucleic acid sequences may also be detected and monitored by lateral flow devices. Lateral Flow devices are well known. These devices generally include a solid phase fluid permeable flow path through which fluid flows through by capillary force. Examples include, but are not limited to, dipstick assays and thin layer chromatographic plates with various appropriate coatings. Immobilized on the flow path are various binding reagents for the sample, binding partners or conjugates involving binding partners for the sample and signal producing systems. Detection of samples can be achieved in several manners; enzymatic detection, nanoparticle detection, colorimetric detection, and fluorescence detection, for example. Enzymatic detection may involve enzyme-labeled probes that are hybridized to complementary nucleic aid targets on the surface of the lateral flow device. The resulting complex can be treated with appropriate markers to develop a readable signal. Nanoparticle detection involves bead technology that may use colloidal gold, latex and paramagnetic nanoparticles. In one example, beads may be conjugated to an anti-biotin antibody. Target sequences may be directly biotinylated, or target sequences may be hybridized to a sequence specific biotinylated probes. Gold and latex give rise to colorimetric signals visible to the naked eye and paramagnetic particles give rise to a non-visual signal when excited in a magnetic field and can be interpreted by a specialized reader.

Fluorescence-based lateral flow detection methods are also known, for example, dual fluorescein and biotin-labeled oligo probe methods, UPT -N ALP utilizing up-converting phosphor reporters composed of lanthanide elements embedded in a crystal (Corstjens et al., Clinical Chemistry, 47:10,1885-1893,2001), as well as the use of quantum dots.

Nucleic acids can also be captured on lateral flow devices. Means of capture may include antibody dependent and antibody independent methods. Antibody-dependent capture generally comprises an antibody capture line and a labeled probe of complementary sequence to the target. Antibody-independent capture generally uses non-covalent interactions between two binding partners, for example, the high affinity and irreversible linkage between a biotinylated probe and a streptavidin line. Capture probes may be immobilized directly on lateral flow membranes. Both antibody dependent and antibody independent methods may be used in multiplexing.

The production or presence of target nucleic acids and nucleic acid sequences may also be detected and monitored by multiplex DNA sequencing. Multiplex DNA sequencing is a means of identifying target DNA sequences from a pool of DNA. The technique allows for the simultaneous processing of many sequencing templates. Pooled multiple templates can be resolved into individual sequences at the completion of processing. Briefly, DNA molecules are pooled, amplified and chemically fragmented. Products are fractionated by size on sequencing gels and transferred to nylon membranes. The membranes are probed and autoradiographed using methods similar to those used in standard DNA sequencing techniques (Church et al., Science 1998 Apr 8;240(4849):185-188). Autoradiographs can be evaluated and the presence of target nucleic acid sequence can be quantitated.

Kits

Kits of the present invention may comprise, for example, one or more polymerases, forward and reverse templates, and one or more nicking enzymes, as described herein. Where one target is to be amplified, one or two nicking enzymes may be included in the kit. Where multiple target sequences are to be amplified, and the templates designed for those target sequences comprise the nicking enzyme sites for the same nicking enzyme, then one or two nicking enzymes may be included. Or, where the templates are recognized by different nicking enzymes, more nicking enzymes may be included in the kit, such as, for example, 3 or more.

The kits of the present invention may also comprise one or more of the components in any number of separate containers, packets, tubes, vials, microtiter plates and the like, or the components may be combined in various combinations in such containers.

The components of the kit may, for example, be present in one or more containers, for

example, all of the components may be in one container, or, for example, the enzymes may be in a separate container from the templates. The components may, for example, be lyophilized, freeze dried, or in a stable buffer. In one example, the polymerase and nicking enzymes are in lyophilized form in a single container, and the templates are either lyophilized, freeze dried, or in buffer, in a different container. Or, in another example, the polymerase, nicking enzymes, and the templates are, in lyophilized form, in a single container. Or, the polymerase and the nicking enzyme may be separated into different containers.

Kits may further comprise, for example, dNTPs used in the reaction, or modified nucleotides, cuvettes or other containers used for the reaction, or a vial of water or buffer for re-hydrating lyophilized components. The buffer used may, for example, be appropriate for both polymerase and nicking enzyme activity.

The kits of the present invention may also comprise instructions for performing one or more methods described herein and/or a description of one or more compositions or reagents described herein. Instructions and/or descriptions may be in printed form and may be included in a kit insert. A kit also may include a written description of an Internet location that provides such instructions or descriptions.

Kits may further comprise reagents used for detection methods, such as, for example, reagents used for FRET, lateral flow devices, dipsticks, fluorescent dye, colloidal gold particles, latex particles, a molecular beacon, or polystyrene beads.

Examples

Example 1: Detection of DNA NEAR assay products by gel electrophoresis

The NEAR amplification reaction products can be visualized by gel electrophoresis. In the absence of target, the templates (with complementary 3' bases) overlap by one or more bases, polymerase extends in each direction to generate the NEAR amplification duplex (Figure IB); and the amplification proceeds in a similar mechanism to the NEAR amplification to amplify a product that is two bases shorter than the target amplified product. In the case of a 25mer assay where the templates end in A and T, the resulting background product is 23 bases. The 27mer assay also forms a 23mer background and 27mer product. Longer reaction products are also amplified. The sequence of these products is hypothesized to be due to the polymerase extension

before the nicking enzyme can nick both sides of the NEAR amplification duplex, according to Steps 9B in Figure Ie. Figure 2 shows the NEAR reaction products are easily distinguished from background products by gel electrophoresis.

Example 2: Detection of RNA NEAR assay products by gel electrophoresis

The NEAR reaction can also amplify RNA targets. In this case, the target is Ebola Armored RNA, which is a -600 base strand of RNA encapsulated by MS2 phage coat proteins to simulate a viral particle. The reaction is designed to amplify a 25-base region of the Ebola genome contained within the encapsulated RNA sequence. Reaction products run on a 20% polyacrylamide gel (Figure 3) show the amplified 25mer product along with 23mer and 20mer background products. This example demonstrates the ability of the NEAR reaction to amplify RNA released from virus-like particles.

Example 3: Detection of DNA and RNA NEAR assay products by mass spectrometry

The NEAR reaction amplification products can also be detected by mass spectrometry using an ESI/TOF system with a front end LC. The reaction products observed are multiple charged ion species. Usually, the -3 or -4 charge state is the major peak in the spectrum (in the range of 1000-3000 AMU), depending on the length of the oligonucleotide product. The sodium adduct is usually present in the spectrum as a peak adjacent to the major peak at roughly 20-25% the intensity. The unique peaks for the positive reactions in the presence of target are visible in both Figures 4 and 5 for the DNA and RNA NEAR reactions respectively. The background products formed in these NEAR reactions are not shown in the mass range of these spectra.

Example 4: Real-time detection of the NEAR assay amplification

The NEAR amplification reaction can also be monitored, as shown in Figure 6, in real-time with SYBR II fluorescence. The fluorescence increases as SYBR II intercalates into the amplified double-stranded products. The background products also generate fluorescence at a slower rate than the true product. Optimization of amplification sequence, reaction temperature and reaction buffer conditions are necessary in order to visualize distinct separation between the positive reactions and the negative controls.

Example 5: FRET detection of real-time NEAR assay amplification

NEAR amplification can also be monitored by Fluorescence Resonance Energy Transfer (FRET), as shown in Figure 7. Amplification occurs using dual labeled templates, one on each end (5-FAM, 3-BHQ). Fluorescence is generated from the FAM-labeled oligonucleotide upon cleavage of the template by the nicking enzyme when it becomes double-stranded. Since fluorescence is produced by the initial nicking reaction, this detection method is extremely responsive. Since the 3' ends of the templates are blocked from extension by the quenching label, the production of background fluorescence is inhibited.

Example 6: Molecular beacon detection of real-time NEAR amplification

A third method of monitoring real-time amplification is using molecular beacons, as shown in Figure 8. In this case, the amplified product hybridizes to the loop region of the molecular beacon resulting in an increase in fluorescence from the separation of the fluorophore and quencher on each end of the hairpin stem. Since this interaction occurs post-amplification, it is considered pseudo-real-time and can be slightly slower in response relative to the FRET approach.

Example 7: False Alarm Rate testing

This experiment was designed to probe the probability that the NEAR amplification reaction will yield a true product in the negative reaction, or a false positive. NEAR reactions directed at specific amplification of a 25mer region specific to the *Bacillus subtilis* genome were run in the presence (n = 120) and absence (n =320) of *Bacillus subtilis* genomic DNA. End point reactions were run on the mass spectrometer and the area under the curve (AUC) for the product mass peak in the mass spectrum was analyzed. As shown in Figure 9, the results show that none of the 320 negative reactions resulted in a false positive with AUC values equal to the water control. The true positive AUC values were at least 3 standard deviations apart from the true negatives. Overall, these results demonstrate the reproducible nature of the NEAR assay.

Example 8: Beacon detection: NEAR assay reproducibility with beacon detection

The molecular beacon detection of NEAR reaction products can also be used as an endpoint reading. As shown in Figure 10, the ratio of NEAR reaction products can be manipulated by varying the input ratio of the forward and reverse templates. Skewing the templates to favor one of the reaction products allows the single-stranded product to be available for hybridization to a molecular beacon. The open beacon generates a fluorescent signal. This detection method is extremely reproducible. In this study, two operators performed replicates of the same assay on two different days. The results of this study demonstrate the reproducibility of the assay from one day to the next as well as reproducibility between operators.

Example 9: NEAR Assay sensitivity with beacon detection

The sensitivity of the NEAR assay with beacon read-out was tested using a dilution of *Francisella tularensis* genomic DNA. As shown in Figure 11, as few as 50 copies were detected above the no target control.

Example 10: Concentration of amplified products for NEAR DNA amplification

The sensitivity of the NEAR assay has also been studied using mass spectrometry detection of the reaction products. Figure 12 shows signal above the no target control down to 100 copies. The data from this study was used to correlate the input copy number to the final amount of amplified product. In this study, the AUC values of the mass spec product peaks were fit to a standard curve to give the estimated final concentration of amplified product for the NEAR assay. The amount of amplified product ranges from approximately 250nM to almost lμM for 1E+2 and 1E+5 copies respectively. This product amount results in a 1E+8 to 7E+ 10-fold amplification. These reactions were performed without the hot-start conditions, in fact hot-start conditions have been shown to dramatically increase the amount of product amplified, so a further increase in amplification is achieved. The zero copy amplification reaction has a positive final concentration due to the y-intercept value in the standard curve equation.

Example 11: Concentration of amplified products for RNA assay

A similar study was performed on the NEAR amplification of RNA. A dilution of RNA targets were amplified by the NEAR assay. Products were run on the mass spec and the AUC

values of the product peaks were analyzed against a standard curve to determine the concentration of the final product, as shown in Figure 13. A 12 minute amplification starting with 30 and 30,000 copies of initial target results in a 3E+9 to 1E+7 -fold amplification respectively. The lower extent of amplification compared to the DNA amplification could be due to the less efficient reverse transcriptase ability of the polymerase compared to its replication abilities. Also, the RNA:DNA hybrid formed upon the extension of the reverse template is a stronger interaction compared to a normal DNA:DNA hybrid and will have less breathing to allow for the forward or another reverse template to displace one strand. However, amplification products from the RNA reaction were detected down to <100 copies.

Example 12: NEAR reaction specificity for DNA

Since the reaction products are usually between 20 and 30 bases in length, the question arises as to whether or not these short amplification assays can be specific enough to target a single sequence region with other near neighbor genomes present. The NEAR reaction was tested for its specificity by running the amplification reaction in the presence and absence of varying amounts of the near neighbor genomic DNA (Figure 14). In this case, the assay detects a specific sequence in the pX02 plasmid of *Bacillus anthracis* and the near neighbor genome is *Bacillus thuringiensis* (kurstaki). The reactions were analyzed by the AUC values for the product peaks. The figure below demonstrates that in the absence of the correct target *(Bacillus anthracis),* there is no true product amplified (the levels are so low that they are not visible on the scale of the graph). The amount of amplification of the positive reactions is consistent, with larger error bars for the 0 and 5E+5 copies of *Bacillus thuringiensis* (5E+4 copies of *Bacillus anthracis)* due to a single lower value for one of the triplicate runs. Overall the experiment demonstrates that the NEAR reaction is very specific to the target sequence when the assay is designed within a unique region of the genome.

Example 13: Interferent testing

A panel of interferents was tested to monitor the effect of each on the NEAR assay amplification. Figure 15 demonstrates the robust nature of the NEAR assay in the presence of interferents. Some interferents that are known to inhibit PCR, such as humic acid, did not appear

to inhibit the NEAR assay, though the amount of each interferent is unknown. From statistical analysis only interferent B, C, and E were statistically different from the control assay x. In the B, C, and E cases, the difference resulted in increased product amplification.

Example 14: Multiplexing of two sequences with NEAR DNA assays

A DNA duplex was designed for capillary electrophoresis (CE) detection. Amplification products were 25 bases *(Bacillus anthracis* assay, *Ba)* and 27 bases *(Bacillus subtilis* assay, *Bs)* in length with background production of a 23mer. The reaction was run for 10 minutes in the presence or absence of 5E+5 copies of the respective genomic DNA target. The samples were run on a 20% polyacrylamide gel to visualize the reaction products. Figure 16 indicates the presence of positive product amplification when *Bacillus subtilis* only is present as well as when both *Bacillus subtilis* and *Bacillus anthracis* are present.

Example 15: NEAR DNA assay duplex specificity

The NEAR DNA duplex reaction with *Bacillus subtilis (Bs)* and *Bacillus anthracis (Ba)* was shown to be specific to the respective genomes. The assays were run in the presence of the near neighbor, *Bacillus thuringiensis,* as shown in Figure 17. In the negative reaction where both template sets are present as well as the *Bacillus thuringiensis* genomic DNA, there is no product band in the 25 or 27mer region. Product bands appear only when the specific genomic target is present, which demonstrates the specificity of the duplex reaction.

Example 16: Multiplexing with NEAR RNA assays

An MS2 assay that amplifies a 27mer product and an Ebola assay that amplifies a 25mer product was developed and multiplexed so that all templates are present in each assay and amplification of products is dependent on the target present. This combination of templates forms background products that are 23 bases and 20 bases in length. The gel shown in Figure 18 demonstrates the ability for the NEAR reaction to amplify multiple RNA targets in a single reaction.

Example 17: Amplification from lysed spores by NEAR Assay

Amplification was performed on semi-processed samples to determine whether it is possible to amplify DNA released from spores through lysis. The negative control reaction contained DNase-treated spores, unlysed, so no DNA should be present to amplify. The positive control reaction contained purified genomic DNA at concentrations around the amount of DNA estimated to be released through lysis. Results in Figure 19 show that amplification with unlysed DNase-treated spores results in no product amplification as expected, whereas the three samples lysed before amplification resulted in product amounts in the range of the theoretical amounts.

Example 18: Capture and Extension

The NEAR reaction products can also be detected on a solid surface. A capture probe attached at the 5' end to the surface through a biotin/streptavidin attachment can bind to the reaction products from which polymerase extends to form a stable duplex that SYBR and any intercalating dye can detect. The capture probe is designed to favor extension through binding to the true product over background products because the 3' base of the capture probe is complementary to the middle spacer base in the product which is not present in either of the templates or the background products. Figure 20 demonstrates the increased fluorescence of the NEAR products in the presence of the capture probe and polymerase over the average binding (same reaction in the absence of polymerase, to preclude extension of the capture probe) and the no target control where only background products are amplified, but cannot form a stable duplex with the capture probe for polymerase to extend.

Example 19: Surface NEAR FRET DNA Assay

The NEAR reaction can also be performed with the templates immobilized on the surface. The templates for FRET detection of surface amplification usually have three modifications: one 5' biotin with a TEG spacer, one FAM fluorophore internal to the biotin, and a quencher on the 3' end which serves to block background amplification as well as to quench the FAM fluorophore. The template is immobilized on the surface through biotin/streptavidin attachment. Figure 21 demonstrates that with both templates immobilized along with additional mixing, the reaction proceeds at a much slower rate than the solution amplification rate (amplification in 16 minutes for lE+6 copies of genomic DNA). When a single template is

immobilized on the surface and the other template is free in solution, the amplification reaction is increased to 10 minute detection for lE+6 copies of genomic DNA. Fluorescence from background products is observed -3.5 minutes after the product signal, similar to what is observed for solution phase kinetics, but slowed considerably.

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The entirety of each patent, patent application, publication and document referenced herein hereby is incorporated by reference. Citation of the above patents, patent applications, publications and documents is not an admission that any of the foregoing is pertinent prior art, nor does it constitute any admission as to the contents or date of these publications or documents.

Singular forms “a”, “an”, and “the” include plural reference unless the context clearly dictates otherwise. Thus, for example, reference to “a subset” includes a plurality of such subsets, reference to “a nucleic acid” includes one or more nucleic acids and equivalents thereof known to those skilled in the art, and so forth. The term “or” is not meant to be exclusive to one or the terms it designates. For example, as it is used in a phrase of the structure “A or B” may denote A alone, B alone, or both A and B.

Unless defined otherwise, all technical and scientific terms used herein have the same meanings as commonly understood by one of ordinary skill in the art to which this invention belongs. Although any methods and systems similar or equivalent to those described herein can be used in the practice or testing of the present invention, the methods, devices, and materials are now described. All publications mentioned herein are incorporated herein by reference for the purpose of describing and disclosing the processes, systems, and methodologies that are reported in the publications which might be used in connection with the invention. Nothing herein is to be construed as an admission that the invention is not entitled to antedate such disclosure by virtue of prior invention.

Modifications may be made to the foregoing without departing from the basic aspects of the invention. Although the invention has been described in substantial detail with reference to one or more specific embodiments, those of ordinary skill in the art will recognize that changes may be made to the embodiments specifically disclosed in this application, and yet these modifications and improvements are within the scope and spirit of the invention. The invention illustratively described herein suitably may be practiced in the absence of any element(s) not

specifically disclosed herein. Thus, for example, in each instance herein any of the terms "comprising", "consisting essentially of", and "consisting of" may be replaced with either of the other two terms. Thus, the terms and expressions which have been employed are used as terms of description and not of limitation, equivalents of the features shown and described, or portions thereof, are not excluded, and it is recognized that various modifications are possible within the scope of the invention. Embodiments of the invention are set forth in the following claims.

NICKING AND EXTENSION AMPLIFICATION REACTION FOR THE EXPONENTIAL AMPLIFICATION OF NUCLEIC ACIDS

Related Applications

This application is a continuation of U.S. Application Serial No. 11/778,018, filed July 14, 2007, the entire contents of which are hereby incorporated.

Field of the Invention

The invention is in general directed to the rapid exponential amplification of short DNA or RNA sequences at a constant temperature.

Background

The field of *in vitro* diagnostics is quickly expanding as the need for systems that can rapidly detect the presence of harmful species or determine the genetic sequence of a region of interest is increasing exponentially. Current molecular diagnostics focus on the detection of biomarkers and include small molecule detection, immuno-based assays, and nucleic acid tests. The built-in specificity between two complementary nucleic acid strands allows for fast and specific recognition using unique DNA or RNA sequences, the simplicity of which makes a nucleic acid test an attractive prospect. Identification of bacterial and viral threat agents, genetically modified food products, and single nucleotide polymorphisms for disease management are only a few areas where the advancement of these molecular diagnostic tools becomes extremely advantageous. To meet these growing needs, nucleic acid amplification technologies have been developed and tailored to these needs of specificity and sensitivity.

Historically, the most common amplification technique is the polymerase chain reaction (PCR), which has in many cases become the gold standard for detection methods because of its reliability and specificity. This technique requires the cycling of temperatures to proceed through the steps of denaturation of the dsDNA, annealing of short oligonucleotide primers, and extension of the primer along the template by a thermostable polymerase. Though many new advances in engineering have successfully shortened these reaction times to 20-30 minutes, there is still a steep power requirement to meet the needs of these thermocycling units.

Various isothermal amplification techniques have been developed to circumvent the need for temperature cycling. From this demand, both DNA and RNA isothermal amplification technologies have emerged.

Transcription-Mediated Amplification (TMA) employs a reverse transcriptase with RNase activity, an RNA polymerase, and primers with a promoter sequence at the 5'end. The reverse transcriptase synthesizes cDNA from the primer, degrades the RNA target, and synthesizes the second strand after the reverse primer binds. RNA polymerase then binds to the promoter region of the dsDNA and transcribes new RNA transcripts which can serve as templates for further reverse transcription. The reaction can produce a billion fold amplification in 20-30 minutes. This system is not as robust as other DNA amplification techniques and is therefore, not a field-deployable test due to the ubiquitous presence of RNAases outside of a sterile laboratory. This amplification technique is very similar to Self-Sustained Sequence Replication (3SR) and Nucleic Acid Sequence Based Amplification (NASBA), but varies in the enzymes employed.

Single Primer Isothermal Amplification (SPIA) also involves multiple polymerases and RNaseH. First, a reverse transcriptase extends a chimeric primer along an RNA target. RNaseH degrades the RNA target and allows a DNA polymerase to synthesize the second strand of cDNA. RNaseH then degrades a portion of the chimeric primer to release a portion of the cDNA and open a binding site for the next chimeric primer to bind and the amplification process proceeds through the cycle again. The linear amplification system can amplify very low levels of RNA target in roughly 3.5 hrs.

The Q-Beta replicase system is a probe amplification method. A probe region complementary to the target of choice is inserted into MDV-l RNA, a naturally occurring template for Q-Beta replicase. Q-Beta replicates the MDV -1 plasmid so that the synthesized product is itself a template for Q-Beta replicase, resulting in exponential amplification as long as the there is excess replicase to template. Because the Q-Beta replication process is so sensitive and can amplify whether the target is present or not, multiple wash steps are required to purge the sample of non-specifically bound replication plasmids. The exponential amplification takes approximately 30 minutes; however, the total time including all wash steps is approximately 4 hours.

Numerous isothermal DNA amplification technologies have been developed as well. Rolling circle amplification (RCA) was developed based on the natural replication of plasmids and viruses. A primer extends along a circular template resulting in the synthesis of a single-stranded tandem repeat. Capture, washing, and ligation steps are necessary to preferentially circularize the template in the presence of target and reduce background amplification. Ramification amplification (RAM) adds cascading primers for additional geometric amplification. This technique involves amplification of non-specifically sized strands that are either double or single-stranded.

Helicase-dependent amplification (HDA) takes advantage of a thermostable helicase (Tte-UvrD) to unwind dsDNA to create single-strands that are then available for hybridization and extension of primers by polymerase. The thermostable HDA method does not require the accessory proteins that the non-thermostable HDA requires. The reaction can be performed at a single temperature, though an initial heat denaturation to bind the primers generates more product. Reaction times are reported to be over 1 hour to amplify products 70-120 base pairs in length.

Loop mediated amplification (LAMP) is a sensitive and specific isothermal amplification method that employs a thermostable polymerase with strand displacement capabilities and four or more primers. The primers are designed to anneal consecutively along the target in the forward and reverse direction. Extension of the outer primers displaces the extended inner primers to release single strands. Each primer is designed to have hairpin ends that, once displaced, snap into a hairpin to facilitate self-priming and further polymerase extension. Additional loop primers can decrease the amplification time, but complicates the reaction mixture. Overall, LAMP is a difficult amplification method to multiplex, that is, to amplify more than one target sequence at a time, although it is reported to be extremely specific due to the multiple primers that must anneal to the target to further the amplification process. Though the reaction proceeds under isothermal conditions, an initial heat denaturation step is required for double-stranded targets. Amplification proceeds in 25 to 50 minutes and yields a ladder pattern of various length products.

Strand displacement amplification (SDA) was developed by Walker et.al. in 1992. This amplification method uses two sets of primers, a strand displacing polymerase, and a restriction

endonuclease. The bumper primers serve to displace the initially extended primers to create a single-strand for the next primer to bind. A restriction site is present in the 5' region of the primer. Thiol-modified nucleotides are incorporated into the synthesized products to inhibit cleavage of the synthesized strand. This modification creates a nick site on the primer side of the strand, which the polymerase can extend. This approach requires an initial heat denaturation step for double-stranded targets. The reaction is then run at a temperature below the melting temperature of the double-stranded target region. Products 60 to 100 bases in length are usually amplified in 30-45 minutes using this method.

These and other amplification methods are discussed in, for example, VanNess, J, et al., PNAS 2003. Vol. 100, no 8, p 4504-4509; Tan, E., et al., Anal. Chem. 2005, 77, 7984-7992; Lizard, P., et al., Nature Biotech. 1998, 6, 1197-1202; Notomi, T., et al., NAR 2000,28,12, e63; and Kurn, N., et al., Clin. Chem. 2005, 51:10,1973-1981. Other references for these general amplification techniques include, for example, U.S. Patent Serial Nos. 7112423; 5455166; 5712124; 5744311; 5916779; 5556751;5733733; 5834202;5354668; 5591609; 5614389; 5942391; and U.S. patent publication numbers US20030082590; US20030138800; US20040058378; and US20060154286.

There is a need for a quicker method of amplification of single-stranded and double-stranded nucleic acid target sequences that can be performed without temperature cycling and that is suitable for shorter target sequences.

Summary

Provided herein are methods of amplifying nucleic acid target sequences that rely on nicking and extension reactions and amplify shorter sequences in a quicker timeframe than traditional amplification reactions, such as, for example, strand displacement amplification reactions. Embodiments of the invention include, for example, reactions that use only two templates to prime, one or two nicking enzymes, and a polymerase, under isothermal conditions. In exemplary embodiments, the polymerase and the nicking enzyme are thermophilic, and the reaction temperature is significantly above the melting temperature of the hybridized target region. The nicking enzyme nicks only one strand in a double-stranded duplex, so that incorporation of modified nucleotides is not necessary as it is in strand displacement. An initial

heat denaturation step is not required for the methods of the present invention. Due to the simplicity of the reaction, in exemplary embodiments, the reaction is very easy to perform and can amplify 20-30mer products 10^8 to 10^{10} fold from genomic DNA in 2.5 to 10 minutes. Furthermore, in other exemplary embodiments, the method is able to amplify RNA without a separate reverse transcription step.

Thus, provided in a first embodiment of the present invention is a method for amplifying a double-stranded nucleic acid target sequence, comprising contacting a target DNA molecule comprising a double-stranded target sequence having a sense strand and an antisense strand, with a forward template and a reverse template, wherein said forward template comprises a nucleic acid sequence comprising a recognition region at the 3' end that is complementary to the 3' end of the target sequence antisense strand; a nicking enzyme site upstream of said recognition region, and a stabilizing region upstream of said nicking enzyme site; said reverse template comprises a nucleotide sequence comprising a recognition region at the 3' end that is complementary to the 3' end of the target sequence sense strand, a nicking enzyme site upstream of said recognition region, and a stabilizing region upstream of said nicking enzyme site; providing a first nicking enzyme that is capable of nicking at the nicking enzyme site of said forward template, and does not nick within said target sequence; providing a second nicking enzyme that is capable of nicking at the nicking enzyme site of said reverse template and does not nick within said target sequence; and providing a DNA polymerase; under conditions wherein amplification is performed by multiple cycles of said polymerase extending said forward and reverse templates along said target sequence producing a double-stranded nicking enzyme site, and said nicking enzymes nicking at said nicking enzyme sites, producing an amplification product.

In certain embodiments of the invention, the DNA polymerase is a thermophilic polymerase. In other examples of the invention, the polymerase and said nicking enzymes are stable at temperatures up to 37°C, 42°C, 60°C, 65°C, 70°C, 75°C, 80°C, or 85°C. In certain embodiments, the polymerase is stable up to 60°C. The polymerase may, for example, be selected from the group consisting of Bst (large fragment), 9°N, VentR ® (exo-) DNA Polymerase, Therminator, and Therminator II.

The nicking enzyme may, for example, nick upstream of the nicking enzyme binding site,

or, in exemplary embodiments, the nicking enzyme may nick downstream of the nicking enzyme binding site. In certain embodiments, the forward and reverse templates comprise nicking enzyme sites recognized by the same nicking enzyme and said first and said second nicking enzyme are the same. The nicking enzyme may, for example, be selected from the group consisting of Nt.BspQI, Nb.BbvCi, Nb.BsmI, Nb.BsrDI, Nb.BtsI, Nt.AlwI, Nt.BbvCI, Nt.BstNBI, Nt.CviPII, Nb.BpulOI, and Nt.Bpul0I.

In certain aspects of the present invention, the target sequence comprises from 1 to 5 nucleotides more than the sum of the nucleotides of said forward template recognition region and said reverse template recognition region.

The DNA molecule may be, for example, genomic DNA. The DNA molecule may be, for example, selected from the group consisting of plasmid, mitochondrial, and viral DNA. In certain embodiments, the forward template is provided at the same concentration as the reverse template. In other examples, the forward template is provided at a ratio to the reverse template at the range of ratios of 1: 100 to 100:1.

In other examples of the invention, the method further comprises the use of a second polymerase. The amplification may be, for example, conducted at a constant temperature. This temperature may be, for example, between 54°C and 60°C. As to the length of time for the reaction to take place, in certain examples, the amplification reaction is held at constant temperature for 1 to 10 minutes.

The present invention further comprises detecting the amplification product, for example, by a method selected from the group consisting of gel electrophoresis, mass spectrometry, SYBR I fluorescence, SYBR II fluorescence, SYBR Gold, Pico Green, TOTO-3, intercalating dye detection, FRET, molecular beacon detection, surface capture, capillary electrophoresis, incorporation of labeled nucleotides to allow detection by capture, fluorescence polarization, and lateral flow capture. The amplification products may be, for example, detected using a solid surface method, for example, where at least one capture probe is immobilized on the solid surface that binds to the amplified sequence.

The present invention may be used for multiplex amplification. Thus, for example, in certain embodiments of the present invention at least two target sequences are capable of being amplified. By "capable of being amplified" is meant the amplification reaction comprises the

appropriate templates and enzymes to amplify at least two target sequences. Thus, for example, the amplification reaction may be prepared to detect at least two target sequences, but only one of the target sequences may actually be present in the sample being tested, such that both sequences are capable of being amplified, but only one sequence is. Or, where two target sequences are present, the amplification reaction may result in the amplification of both of the target sequences. The multiplex amplification reaction may result in the amplification of one, some, or all, of the target sequences for which it comprises the appropriate templates and enzymes.

At least one of the templates, for example, may comprise a spacer, a blocking group, or a modified nucleotide.

Also provided as an embodiment of the present invention is a method for amplifying a single-stranded nucleic acid target sequence, comprising contacting a target nucleic acid comprising a single-stranded target sequence with a reverse template, wherein said reverse template comprises a nucleotide sequence comprising a recognition region at the 3' end that is complementary to the 3' end of the target sequence, a nicking enzyme site upstream of said recognition region, and a stabilizing region upstream of said nicking enzyme site; providing a first nicking enzyme that is capable of nicking at the nicking enzyme site of said reverse template, and does not nick within said target sequence; providing a DNA polymerase under conditions wherein said polymerase extends said reverse template along said target sequence; contacting said extended reverse template with a forward template, wherein said forward template comprises comprising a recognition region at the 3' end that is identical to the 5' end of the target sequence a nicking enzyme site upstream of said recognition region, and a stabilizing region upstream of said nicking enzyme site; providing a second nicking enzyme that is capable of nicking at the nicking enzyme site of said forward template and does not nick within said target sequence; under conditions wherein amplification is performed by multiple cycles of said polymerase extending said forward and reverse templates along said target sequence producing a double-stranded nicking enzyme site, and said nicking enzymes nicking at said nicking enzyme sites, producing an amplification product.

Those of ordinary skill in the art understand that the examples presented herein relating to the amplification of a double-stranded nucleic acid target sequence and the detection of the

amplified product also apply to the amplification of a single-stranded nucleic acid target sequence and the detection of the amplified product. Further, in examples of the present invention, the target sequence may be, for example, RNA, for example, but not limited to, messenger RNA, viral RNA, microRNA, a microRNA precursor, or siRNA. In exemplary embodiments of the present invention, the polymerase comprises reverse transcription activity. In yet other examples of the present invention, the target sequence is DNA, such as, for example, genomic DNA, or for example, the target sequence is selected from the group consisting of plasmid, mitochondrial, and viral nucleic acid.

Where the method may comprise the use of more than one polymerase, in exemplary embodiments at least one of the polymerases comprises reverse transcriptase activity.

In other embodiments of the present invention, a set of oligonucleotide templates is provided, comprising a first template for nucleic acid amplification, comprising a recognition region at the 3' end that is complementary to the 3' end of a target sequence antisense strand; a nicking enzyme site upstream of said recognition region; and a stabilizing region upstream of said nicking enzyme site; and a second template for nucleic acid amplification, comprising a recognition region at the 3' end that is identical to the 5' of said target sequence antisense strand; a nicking enzyme site upstream of said recognition region; and a stabilizing region upstream of said nicking enzyme site; wherein said target sequence comprises from 1 to 5 spacer bases between said 3' end of the antisense strand and said 5' end of said antisense strand that do not bind to either template.

In yet other embodiments, a kit is provided for following the methods of the present invention for nucleic acid amplification, comprising a DNA polymerase; a first template for nucleic acid amplification, comprising a recognition region at the 3' end that is complementary to the 3' end of a target sequence antisense strand; a nicking enzyme site upstream of said recognition region; and a stabilizing region upstream of said nicking enzyme site; a second template for nucleic acid amplification, comprising a recognition region at the 3' end that is complementary to the 3' end of a target sequence sense strand; a nicking enzyme site upstream of said recognition region; and a stabilizing region upstream of said nicking enzyme site; one or two thermostable nicking enzymes, wherein either one enzyme is capable of nicking at the nicking enzyme site of said first and said second templates, or a first enzyme is capable of

nicking at the nicking enzyme site of said first primer and a second enzyme is capable of nicking at the enzyme site of said second primer.

The kit may, for example, provide said polymerase, nicking enzymes, and templates in a container. The kit may provide, for example, said polymerase, nicking enzymes, and templates in two containers. In certain examples, the polymerase and nicking enzymes are in a first container, and said templates are in a second container. In certain examples, the polymerase and nicking enzymes are lyophilized. The kit may, for example, further comprise instructions for following the amplification methods of the present invention. The kit may, for example, further comprise a cuvette. The kit may, for example, further comprise a lateral flow device or dipstick. The lateral flow device or dipstick may, for example, further comprise a capture probe, wherein said capture probe binds to amplified product. The kit may, for example, further comprise a detector component selected from the group consisting of a fluorescent dye, colloidal gold particles, latex particles, a molecular beacon, and polystyrene beads. In other examples, at least one of the templates of the kit comprises a spacer, blocking group, or a modified nucleotide.

Deoxynucleoside triphosphates (dNTPs) are included in the amplification reaction. One or more of the dNTPs may be modified, or labeled, as discussed herein. Nucleotides are designated as follows. A ribonucleoside triphosphate is referred to as NTP or rNTP; N can be A, G, C, U or m5U to denote specific ribonucleotides. Deoxynucleoside triphosphate substrates are indicated as dNTPs, where N can be A, G, C, T, or U. Throughout the text, monomeric nucleotide subunits may be denoted as A, G, C, or T with no particular reference to DNA or RNA.

In another embodiment, a method is provided for nucleic acid amplification comprising forming a mixture of a target nucleic acid comprising a double-stranded target sequence having a sense strand and an antisense strand; a forward template comprising a nucleic acid sequence comprising a recognition region at the 3' end that is complementary to the 3' end of the target sequence antisense strand; a nicking enzyme site upstream of said recognition region, and a stabilizing region upstream of said nicking enzyme site; a reverse template comprising a nucleotide sequence comprising a recognition region at the 3' end that is complementary to the 3' end of the target sequence sense strand, a nicking enzyme site upstream of said recognition region and a stabilizing region upstream of said nicking enzyme site; a first nicking enzyme that

is capable of nicking at the nicking enzyme site of said forward template, and does not nick within said target sequence; a second nicking enzyme that is capable of nicking at the nicking enzyme site of said reverse template and does not nick within said target sequence; and a thermophilic polymerase under conditions wherein amplification is performed by multiple cycles of said polymerase extending said forward and reverse templates along said target sequence producing a double-stranded nicking enzyme site, and said nicking enzymes nicking at said nicking enzyme sites, producing an amplification product. In certain embodiments, the nicking enzyme sites on the forward and reverse templates are recognized by the same nicking enzyme, and only one nicking enzyme is used for the reaction.

In another embodiment, a method is provided for nucleic acid amplification comprising forming a mixture of a target nucleic acid comprising a single-stranded target sequence; a reverse template, wherein said reverse template comprises a nucleotide sequence comprising a recognition region at the 3' end that is complementary to the 3' end of the target sequence, a nicking enzyme site upstream of said recognition region, and a stabilizing region upstream of said nicking enzyme site; a first nicking enzyme that is capable of nicking at the nicking enzyme site of said reverse template, and does not nick within said target sequence; a thermophilic polymerase under conditions wherein said polymerase extends said reverse template along said target sequence; a forward template, wherein said forward template comprises a nucleic acid sequence comprising a recognition region at the 3' end that is identical to the 5'end of the target sequence; and a second nicking enzyme that is capable of nicking at the nicking enzyme site of said forward template and does not nick within said target sequence; under conditions wherein amplification is performed by multiple cycles of said polymerase extending said forward and reverse templates along said target sequence producing a double-stranded nicking enzyme site, and said nicking enzymes nicking at said nicking enzyme sites, producing an amplification product. In certain embodiments, the nicking enzyme sites on the forward and reverse templates are recognized by the same nicking enzyme, and only one nicking enzyme is used for the reaction.

In other embodiments of the invention are provided methods for the separation of amplified nucleic acids obtained by the amplification methods of the invention. In yet further embodiments of the invention are provided methods for detecting and/or analyzing the amplified

nucleic acids obtained by the amplification methods of the invention, including, for example, methods using SYBR I, II, SYBR Gold, Pico Green, TOTO-3, and most intercalating dyes, molecular beacons, FRET, surface capture using immobilized probes with fluorescence, electrochemical, or colorimetric detection, mass spectrometry, capillary electrophoresis, the incorporation of labeled nucleotides to allow detection by capture or fluorescence polarization, lateral flow, and other methods involving capture probes. Methods using capture probes for detection include, for example, the use of a nucleic acid molecule (the capture probe) comprising a sequence that is complementary to the amplified product such that the capture probe binds to amplified nucleic acid. The reaction may, for example, further comprise an antibody directed against a molecule incorporated into or attached to the capture probe. Or, for example, the capture probe, or a molecule that binds to the capture probe, may incorporate, for example, an enzyme label, for example, peroxidase, alkaline phosphatase, or beta-galactosidase, a fluorescent label, such as, for example, fluorescein or rhodamine, or, for example, other molecules having chemiluminescent or bioluminescent activity. The embodiments of the present invention also comprise combinations of these detection and analysis methods.

Brief Description of the Drawings

Figures 1A-D are graphic drawings depicting mechanisms of the reactions of the present invention. Figure 1D is a legend for Figure 1.

Figure 2. 20% polyacrylamide gel of reaction products from a DNA NEAR assay.

The NEAR reaction was run for 2.5 minutes at 56°C, then heat denatured at 94 °C for 4 minutes. Six µL of the reaction was run on a 20% polyacrylamide gel at 160V for ~2.5 hrs. The gel was stained with SYBR II gel stain. Lane 1: NEAR reaction no target control for 25mer assay. Lane 2: NEAR reaction no target control for 27mer assay. Lane 3: NEAR reaction for 25mer assay with 3.5E+5 copies of genomic *Bacillus subtilis* DNA. Lane 4: NEAR reaction for 27mer assay with 1.1E+6 copies of genomic *Bacillus subtilis* DNA.

Figure 3. 20% polyacrylamide gel of reaction products from an RNA NEAR assay.

The NEAR reaction was run for 12 minutes at 56°C, then heat denatured at 94 °C for 4 minutes. Six µL of the reaction was run on a 20% polyacrylamide gel at 160V for -2.5 hrs. The gel was stained with SYBR II gel stain. Lane 1 & 2: NEAR reaction for 25mer assay with lE+6

copies of Ebola Armored RNA (Ambion). Lane 3 & 4: NEAR reaction no target control for 25mer assay. 25mer reaction products are outlined in the white box.

Figures 4A-B ~~**Figure 4.**~~ Mass Spectrum of *Bacillus anthracis* DNA assay products.

A) 0 copies of target or B) 5E+5 copies of genomic DNA added to the NEAR reaction. The NEAR reaction was run for 10 minutes, then heat denatured at 94°C for 4 minutes. Ten micro liters of sample was injected into the LC/ESI-MS. The (-4) charge state of the 26mer product and its complementary sequence are outlined in a black box. The smaller adjacent peaks are the sodium adducts of the main product.

Figures 5A-C ~~**Figure 5.**~~ Mass Spectrum of MS2 genomic RNA assay products.

A) 0 copies of target, B) lE+6 copies of MS2 genomic RNA, or C) lE+6 copies of synthetic target DNA added to the NEAR reaction. The NEAR reaction was run for 10 minutes, then heat denatured at 94°C for 4 minutes. Ten micro liters of sample was injected into the LC/ESI-MS. The (-4) charge state of the 27mer product and its complement sequence are outlined in a black box. The smaller adjacent peaks are the sodium adducts of the main product.

Figure 6. Real-time detection of NEAR assay amplification using intercalating fluorescent dyes.

Real-time amplification of *Yersinia pestis* genomic DNA at 500 copies (squares) compared to the no target control (NTC, open triangles). The reaction was run for 10 minutes at 58°C and monitored by the real-time fluorescence with SYBR II (n =5).

Figure 7. Real-time detection of NEAR assay amplification using fluorescence resonance energy transfer (FRET).

Real-time amplification of *Yersinia pestis* synthetic DNA at 10,000 copies (squares) compared to the no target control (NTC, open triangles). The reaction was run for 10 minutes at 57°C, n =3.

Figure 8. Francisella *tularensis* assay amplification detected in real-time using molecular beacons.

Either 0 copies (open triangles) or 1E+5 copies (squares) were added to the reaction mix and run for 10 minutes at 57.5 °C.

Figure 9. False alarm rate testing results comparing average *AVC* values.

Error bars denote one standard deviation. *Bacillus subtilis* NEAR assays were run for 10

min at 55°C in the presence and absence of *Bacillus subtilis* genomic DNA. Enzymes were heat denatured at 94°C for 4 min. A 10 µL sample was injected into the LC/ESI-MS and the area under the *curve (AUC)* of the product peaks were analyzed. True Positives contained 10,000 copies of *Bacillus subtilis* along with 990,000 copies of near neighbor *(Bacillus thuringiensis).* True Negatives contained 10,000 copies of *E. coli* with 990,000 copies of near neighbor, and water negatives contained no DNA as a control.

Figure 10. Replication study of the NEAR Assay using molecular beacon detection with different operators performing the experiments on two different days.

The NEAR reaction was run for 10 minutes at 57.5 °C (in the presence and absence of 500 copies of *Francisella tularensis* genomic DNA) with a 4 min heat kill at 94°C. 300nM molecular beacon was added and monitored at 45, 50, and 57°C (n = 24).

Figure 11. Sensitivity of the NEAR reaction using molecular beacon detection.

The NEAR assay was run for 10 minutes 57.5°C. The reaction was stopped with a 4 min heat denaturation step at 94°C. 300nM molecular beacon was added and the fluorescence was monitored at 57.5°C (n =3). Fluorescence was monitored for beacon opening in the presence NEAR reactions amplified with 1E+6, 5E+5, 5E+4, 5E+2, 50, and 0 (NTC) input copies of *Francisella tularensis* genomic DNA, and compared to the background fluorescence of the beacon alone (MB).

Figure 12. Final concentration of amplified products in the NEAR reaction.

The NEAR reaction was run for 10 min at 55°C with varying copies of *Bacillus subtilis* genomic DNA. The reaction was stopped with a heat denaturation step at 94°C for 4 minutes. A 10 µL sample was injected into the LC/ESI-MS and the AUC of the product peak at 1944 Daltons was analyzed and compared to a standard curve.

Figure 13. Correlation of the input RNA target copy number to the final concentration of amplified products.

The Ebola NEAR assay was run for 12 min at 55°C with varying copies of synthetic RNA corresponding to the Ebola genome DNA. The reaction was stopped with a heat denaturation step at 94°C for 4 minutes. A 10 µL sample was injected into the LC/ESI-MS and the AUC of the product peak at 1936 Daltons was analyzed and compared to the standard curve of AUC values. (n =3)

Figure 14. Mass spec product analysis demonstrating NEAR reaction specificity.

The *Bacillus anthracis* NEAR reaction was run in the presence of a dilution of copies of Bacillus thuringiensis for 10 min at 56°C (n =3), then heat denatured at 94°C for 4 minutes. A 10 µL sample was injected into the LC/ESI-MS and *AUC* values of product peaks analyzed.

Figure 15. The effect of an interferent panel on the NEAR amplification.

Bacillus subtilis NEAR DNA reactions were run for 10 min at 55°C and heated to 94°C for 4 minutes to stop the reaction. Reactions were run in triplicate in the presence 1E+5 copies of *Bacillus subtilis* genomic DNA ("_1E+5") or with no target DNA present ("_0"). Sample x is the control assay with no interferent added. Interferents A through F were added at 50% reaction volume to the *Bacillus subtilis* assay. The *AUC* of mass spec product peaks were analyzed using a two-way ANOV A and Bonferroni t-test. (Key: A =none; B =House dust, skim milk; e =AZ test dust, humic acid; D =Diesel soot; E =Skim milk; F =Mold spores)

Figure 16. Gel electrophoresis results for the *Bacillus subtilis* / *Bacillus anthracis* DNA duplex reaction.

The NEAR reaction including templates for both *Bacillus subtilis (Bs)* and *Bacillus anthracis (Ba)* assays was run in the absence of target DNA (negative), in the presence of *Bacillus subtilis* only (positive for 27mer product), and in the presence of both *Bacillus subtilis* and *Bacillus anthracis* (positive for 27mer and 25mer product respectively). The target copy number used in this assay was 500,000 copies. The assay was run for 10 min at 57°C. Templates varied in concentration between the assays to control the amplification (100nM for *Bacillus anthracis* and 50 nM for *Bacillus subtilis).* Samples were run on a 20% polyacrylamide gel at 160 V for ~2 hours. The gel was stained with SYBR II fluorescent dye and imaged. The fluorescent bands were quantitated and analyzed as the integrated optical density (IOD) (n =8).

Figure 17. Specificity results for the *Bacillus subtilis* / *Bacillus anthracis* DNA duplex reaction shown by gel electrophoresis.

The NEAR reaction including templates for both a *Bacillus subtilis (Bs)* and *Bacillus anthracis (Ba)* assay was run in the absence of target DNA (negative), in the presence of *Bacillus subtilis* only (27mer product), and in the presence of both *Bacillus subtilis* and *Bacillus anthracis* (27mer and 25mer product respectively). The target copy number for each genome present in this assay was 500,000 copies. All reactions contained 500,000 copies of *Bacillus thuringiensis* as

clutter. Templates varied in concentration between the assays to control the amplification. The assay was run for 10 min at 57 °C, heat denatured at 94 °C for 4 min, and 6 μL was loaded on to a 20% gel run at 160 V for ~2 hours. The gel was stained with SYBR II fluorescent dye and imaged. The fluorescent bands were quantitated and analyzed as the integrated optical density (IOD).

Figure 18. Gel electrophoresis results for the MS2/Eboia RNA duplex reaction.

The NEAR reaction including templates for both a MS2 and Ebola assay was run in the absence of target RNA (negative, lanes 2-5), in the presence of MS2 only (27mer product, lanes 6 and 7), and in the presence of both MS2 and Ebola RNA (27mer and 25mer product respectively, lanes 8 and 9). The target copy number used in this assay was 1E+6 copies. The assay was run for 10 min at 57°C. Templates varied in concentration between the assays to control the amplification. Samples were run on a 20% polyacrylamide gel at 160 V for ~2.5 hours. The gel was stained with SYBR II fluorescent dye and imaged. The fluorescent bands were quantitated and analyzed as the integrated optical density (IOD).

Figure 19. Mass spec analysis of NEAR amplification of DNA from lysed spores.

Average AUC values from amplified product masses compared for lysed and unlysed

samples. Lysed spore samples were then added to NEAR master mix and run for 10 minutes at 55°C, heat denatured for 4 minutes at 94 °C, and run on the mass spec for analysis. AUC values of product peaks were averaged and compared (n =3).

Figure 20. Demonstration of the capture and extension approach for surface detection of the NEAR assay.

A.) Average binding (NEAR positive reaction product with no added polymerase), B.) 500,000 target (NEAR positive reaction product with added polymerase), and C.) No target (NEAR negative reaction with added polymerase) are compared. The NEAR assay was run for 10 minutes at 55°C, heat denatured at 94 °C for 4 minutes, then added to the plate with capture probe bound to the surface on the 5' end. Polymerase is added to one well of the positive reaction. The plate is incubated at 55°C for 30 min, washed, SYBR II added, washed 3 times, and read on a Tecan plate reader (495 nm excitation/530 nm emission).

Figure 21. Pseudo-real-time fluorescence detection of the NEAR FRET assay with a single template immobilized on a surface in the presence (squares) and absence (open triangles)

of 1E+6 copies of genomic DNA.

NEAR reaction was performed in flat bottom 96-well plates covered with neutravidin. Solution of 1 μM FRET-labeled reverse template was incubated with gentle mixing for 1 hr at 37 °C. Wells were washed 3 times with a PBS-Tween solution to release unbound template. NEAR reaction mix was added to the wells (one for each time point taken) and incubated at 58°C on a heating block in a shaking incubator set to 135 RPM. Time points were taken by adding 1 μL EDTA to the well to stop the reaction. The fluorescence was read from the bottom using a Tecan 100 plate reader.

Detailed Description

Provided herein are methods for the exponential amplification of short DNA or RNA sequences.

Target nucleic acids of the present invention include double-stranded and single-stranded nucleic acid molecules. The nucleic acid may be, for example, DNA or RNA. Where the target nucleic acid is an RNA molecule, the molecule may be, for example, double-stranded, single-stranded, or the RNA molecule may comprise a target sequence that is single-stranded. Target nucleic acids include, for example, genomic, plasmid, mitochondrial, cellular, and viral nucleic acid. The target nucleic acid may be, for example, genomic, chromosomal, plasmid DNA, a gene, any type of cellular RNA, or a synthetic oligonucleotide. By "genomic nucleic acid" is meant any nucleic acid from any genome, for example, including animal, plant, insect, and bacterial genomes, including, for example, genomes present in spores. Target nucleic acids further include microRNAs and siRNAs.

MicroRNAs, miRNAs, or small temporal RNAs (stRNAs), are short single-stranded RNA sequences, about 21-23 nucleotides long that are involved in gene regulation. MicroRNAs are thought to interfere with the translation of messenger RNAs as they are partially complementary to messenger RNAs. (see, for example, Ruvkun, GI, Science 294:797-99 (2001); Lagos-Quintana, M., et al., Science 294:854-58 (2001); Lau, N.C., et al, Science 294:858-62 (2001); Lee, R.C., and Ambros, V., Science 294:862-64 (2001); Baulcombe, D., et al., Science 297:2002-03 (2002); Llave, c., Science 297:2053-56 (2002); Hutvagner, G., and Zamore, P.D., Science 297:2056-60 (2002)). MicroRNA may also have a role in the immune system, based on

studies recently reported in knock-out mice. (see, for example, Wade, N., "Studies Reveal and Immune System Regulator" New York Times, April 27, 2007). MicroRNA precursors that may also be detected using the methods of the present invention include, for example, the primary transcript (pri-miRNA) and the pre-miRNA stem-loop-structured RNA that is further processed into miRNA.

Short interfering RNAs, or siRNAs are at least partially double-stranded, about 20-25 nucleotide long RNA molecules that are found to be involved in RNA interference, for example, in the down-regulation of viral replication or gene expression (see for example Zamore et al., 2000, Cell, 101,25-33; Bass, 2001, Nature, 411, 428-429; Elbashir et al., 2001, Nature, 411, 494-498; and Kreutzer et al., International PCT Publication No. WO 00/44895; Zernicka-Goetz et al., International PCT Publication No. WO 01/36646; Fire, International PCT Publication No. WO 99/32619; Plaetinck et al., International PCT Publication No. WO 00101846; Mello and Fire, International PCT Publication No. WO 01/29058; Deschamps-Depaillette, International PCT Publication No. WO 99107409; and Li et al., International PCT Publication No. WO 00/44914; Allshire, 2002, Science, 297,1818-1819; Volpe et al., 2002, Science, 297,1833-1837; Jenuwein, 2002, Science, 297, 2215-2218; and Hall et al., 2002, Science, 297, 2232-2237; Hutvagner and Zamore, 2002, Science, 297, 2056-60; McManus et al., 2002, RNA, 8, 842-850; Reinhart et al., 2002, Gene & Dev., 16, 1616-1626; and Reinhart & Bartel, 2002, Science, 297, 1831).

The use of the term "target sequence" may refer to either the sense or antisense strand of the sequence, and also refers to the sequences as they exist on target nucleic acids, amplified copies, or amplification products, of the original target sequence. The amplification product may be a larger molecule that comprises the target sequence, as well as at least one other sequence, or other nucleotides. The length of the target sequence, and the guanosine:cytosine (GC) concentration (percent), is dependent on the temperature at which the reaction is run; this temperature is dependent on the stability of the polymerases and nicking enzymes used in the reaction. Those of ordinary skill in the art may run sample assays to determine the appropriate length and GC concentration for the reaction conditions. For example, where the polymerase and nicking enzyme are stable up to 60°C, then the target sequence may be, for example, from 19 to 50 nucleotides in length, or for example, from 20 to 45, 20 to 40, 22-35, or 23 to 32 nucleotides in length. The GC concentration under these conditions may be, for example, less than 60%, less

than 55%, less than 50%, or less than 45%. The target sequence should not contain nicking sites for any nicking enzymes that will be included in the reaction mix.

The target sequences may be amplified in many types of samples including, but not limited to samples containing spores, viruses, cells, nucleic acid from prokaryotes or eukaryotes, or any free nucleic acid. For example, the assay can detect the DNA on the outside of spores without the need for lysis. The sample may be isolated from any material suspected of containing the target sequence. For example, for animals, for example, mammals, such as, for example, humans, the sample may comprise blood, bone marrow, mucus, lymph, hard tissues, for example, liver, spleen, kidney, lung, or ovary, biopsies, sputum, saliva, tears, feces, or urine. Or, the target sequence may be present in air, plant, soil, or other materials suspected of containing biological organisms.

Target sequences may be present in samples that may also contain environmental and contaminants such as dust, pollen, and diesel exhaust, or clinically relevant matrices such as urine, mucus, or saliva. Target sequences may also be present in waste water, drinking water, air, milk, or other food. Depending on the concentration of these contaminants, sample purification methods known to those of ordinary skill in the art may be required to remove inhibitors for successful amplification. Purification may, for example, involve the use of detergent lysates, sonication, vortexing with glass beads, or a French press. This purification could also result in concentration of the sample target. Samples may also, for be further purified, for example, by filtration, phenol extraction, chromatography, ion exchange, gel electrophoresis, or density dependent centrifugation. The sample can be added directly to the reaction mix or pre-diluted and then added.

An oligonucleotide is a molecule comprising two or more deoxyribonucleotides or ribonucleotides, for example, more than three. The length of an oligonucleotide will depend on how it is to be used. The oligonucleotide may be derived synthetically or by cloning.

The term “complementary” as it refers to two nucleic acid sequences generally refers to the ability of the two sequences to form sufficient hydrogen bonding between the two nucleic acids to stabilize a double-stranded nucleotide sequence formed by hybridization of the two nucleic acids.

As used herein, “hybridization” and “binding” are used interchangeably and refer to the

non-covalent binding or "base pairing" of complementary nucleic acid sequences to one another. Whether or not a particular probe remains base paired with a polynucleotide sequence depends on the degree of complementarity, the length of the probe, and the stringency of the binding conditions. The higher the stringency, the higher must be the degree of complementarity, and/or the longer the probe for binding or base pairing to remain stable.

As used herein, "stringency" refers to the combination of conditions to which nucleic acids are subjected that cause double-stranded nucleic acid to dissociate into component single strands such as pH extremes, high temperature, and salt concentration. The phrase "high stringency" refers to hybridization conditions that are sufficiently stringent or restrictive such that only specific base pairings will occur. The specificity should be sufficient to allow for the detection of unique sequences using an oligonucleotide probe or closely related sequence under standard Southern hybridization protocols (as described in J. Mol. Biol. 98:503 (1975)).

Templates are defined as oligonucleotides that bind to a recognition region of the target and also contain a nicking enzyme binding region upstream of the recognition region and a stabilizing region upstream to the nicking enzyme binding region.

By "recognition region" is meant a nucleic acid sequence on the template that is complementary to a nucleic acid sequence on the target sequence. By "recognition region on the target sequence" is meant the nucleotide sequence on the target sequence that is complementary to, and binds to, the template.

By "stabilizing region" is meant a nucleic acid sequence having, for example, about 50% GC content, designed to stabilize the molecule for, for example, the nicking and/or extension reactions.

In describing the positioning of certain sequences on nucleic acid molecules, such as, for example, in the target sequence, or the template, it is understood by those of ordinary skill in the art that the terms "3'" and "5'" refer to a location of a particular sequence or region in relation to another. Thus, when a sequence or a region is 3' to or 3' of another sequence or region, the location is between that sequence or region and the 3' hydroxyl of that strand of nucleic acid. When a location in a nucleic acid is 5' to or 5' of another sequence or region, that means that the location is between that sequence or region and the 5' phosphate of that strand of nucleic acid.

The polymerase is a protein able to catalyze the specific incorporation of nucleotides to

extend a 3' hydroxyl terminus of a primer molecule, such as, for example, the template oligonucleotide, against a nucleic acid target sequence. The polymerase may be, for example, thermophilic so that it is active at an elevated reaction temperature. It may also, for example, have strand displacement capabilities. It does not, however, need to be very processive (30-40 nucleotides for a single synthesis is sufficient). If the polymerase also has reverse transcription capabilities (such as Bst (large fragment), 9°N, Therminator, Therminator II, etc.) the reaction can also amplify RNA targets in a single step without the use of a separate reverse transcriptase. More than one polymerase may be included in the reaction, in one example one of the polymerases may have reverse transcriptase activity and the other polymerase may lack reverse transcriptase activity. The polymerase may be selected from, for example, the group consisting of one or more of the polymerases listed in Table 1.

Table 1

Polymerase
Bst DNA polymerase
Bst DNA polymerase (Large fragment)
9°Nm DNA polymerase
Phi29 DNA polymerase
DNA polymerase I *(E.coli)*
DNA polymerase I, Large (Klenow) fragment
Klenow fragment (3'-5' exo-)
T4 DNA polymerase
T7 DNA polymerase
Deep VentR™ (exo-) DNA Polymerase
Deep VentR™DNA Polymerase
DyNAzyme™ EXT DNA
DyNAzyme™ II Hot Start DNA Polymerase
Phusion™ High-Fidelity DNA Polymerase
Therminator™ DNA Polymerase
Therminator™ II DNA Polymerase
VentR®DNA Polymerase
VentR® (exo-) DNA Polymerase
RepliPHFM Phi29 DNA Polymerase
rBst DNA Polymerase, Large Fragment (IsoTherm™ DNA Polymerase)
MasterAmpTM AmpliThermTM DNA Polymerase
Tag DNA polymerase
Tth DNA polymerase
Tfl DNA polymerase
Tgo DNA polymerase
SP6 DNA polymerase
Tbr DNA polymerase
DNA polymerase Beta
ThermoPhi DNA polymerase

"Nicking" refers to the cleavage of only one strand of the double-stranded portion of a fully or partially double-stranded nucleic acid. The position where the nucleic acid is nicked is referred to as the nicking site or nicking enzyme site. The recognition sequence that the nicking

enzyme recognizes is referred to as the nicking enzyme binding site. "Capable of nicking" refers to an enzymatic capability of a nicking enzyme.

The nicking enzyme is a protein that binds to double-stranded DNA and cleaves one strand of a double-stranded duplex. The nicking enzyme may cleave either upstream or downstream of the binding site, or nicking enzyme recognition site. In exemplary embodiments, the reaction comprises the use of nicking enzymes that cleave or nick downstream of the binding site (top strand nicking enzymes) so that the product sequence does not contain the nicking site. Using an enzyme that cleaves downstream of the binding site allows the polymerase to more easily extend without having to displace the nicking enzyme. The nicking enzyme must be functional in the same reaction conditions as the polymerase, so optimization between the two ideal conditions for both is necessary. Nicking enzymes are available from, for example, New England Biolabs (NEB) and Fermentas. The nicking enzyme may, for example, be selected from the group consisting of one or more of the nicking enzymes listed in Table 2.

Table 2

Nicking Enzyme	**Alternate Name**
Nb.BbvCI	
Nb.Bpu101	
Nb.Bsal	
Nb.Bsml	
Nb.BsrDI	
Nb.BstNBIP	
Nb.BstSEIP	
Nb.Btsl	
Nb.Sapl	
Nt.Alwl	
Nt.BbvCI	
Nt.BhaIIiP	
Nt.Bpu101	
Nt.Bpu10IB	
Nt.Bsal	
Nt.BsmAI	
Nt.BsmBI	
Nt.BspD61	
Nt.BspQI	
Nt.Bst91	
Nt.BstNBI	N.BstNB I
Nt.BstSEI	
Nt.CviARORFMP	
Nt.CviFRORFAP	
Nt.CviPIl	Nt.CviPlim
Nt.CviQII	
Nt.CviQXI	
Nt.EsaSS1198P	
Nt.Mlyl	
Nt.Sapl	

Nicking enzymes may be, for example, selected from the group consisting of Nt.BspQI(NEB), Nb.BbvCI(NEB), Nb.BsmI(NEB), Nb.BsrDI(NEB), Nb.BtsI(NEB), Nt.AlwI(NEB), Nt.BbvCI(NEB), Nt.BstNBI(NEB), Nt.CviPII(NEB), Nb.BpulOl(Fermantas), and Nt.BpulOl(Fermentas). In certain embodiments, the nicking enzyme is selected from the group consisting of Nt.NBst.NBI, Nb.BsmI, and Nb.BsrDI. Those of ordinary skill in the art are

aware that various nicking enzymes other than those mentioned specifically herein may be used in the methods of the present invention.

Nicking enzymes and polymerases of the present invention may be, for example, stable at room temperature, the enzymes may also, for example, be stable at temperatures up to 37°C, 42°C, 60°C, 65°C, 70°C, 75°C, 80°C, or 85°C. In certain embodiments, the enzymes are stable up to 60°C.

Product or amplified product is defined as the end result of the extension of the template along the target that is nicked, released, and then feeds back into the amplification cycle as a target for the opposite template.

A "native nucleotide" refers to adenylic acid, guanylic acid, cytidylic acid, thymidylic acid, or uridylic acid. A "derivatized nucleotide" is a nucleotide other than a native nucleotide.

The reaction may be conducted in the presence of native nucleotides, such as, for example, dideoxyribonucleoside triphosphates (dNTPs). The reaction may also be carried out in the presence of labeled dNTPs, such as, for example, radiolabels such as, for example, ^{32}p, ^{33}p, ^{125}1, or ^{35}S, enzyme labels such as alkaline phosphatase, fluorescent labels such as fluorescein isothiocyanate (FITC), biotin, avidin, digoxigenin, antigens, haptens, or fluorochromes. These derivatized nucleotides may, for example, be present in the templates.

By "constant temperature," "isothermal conditions" or "isothermally" is meant a set of reaction conditions where the temperature of the reaction is kept essentially constant during the course of the amplification reaction. An advantage of the amplification method of the present invention is that the temperature does not need to be cycled between an upper temperature and a lower temperature. The nicking and the extension reaction will work at the same temperature or within the same narrow temperature range. However, it is not necessary that the temperature be maintained at precisely one temperature. If the equipment used to maintain an elevated temperature allows the temperature of the reaction mixture to vary by a few degrees, this is not detrimental to the amplification reaction, and may still be considered to be an isothermal reaction.

The term "multiplex amplification" refers to the amplification of more than one nucleic acid of interest. For example, it can refer to the amplification of multiple sequences from the same sample or the amplification of one of several sequences in a sample as discussed, for

example, in U.S. Patent Nos. 5,422,252; and 5,470,723, which provide examples of multiplex strand displacement amplification. The term also refers to the amplification of one or more sequences present in multiple samples either simultaneously or in step-wise fashion.

Template Design

Forward and Reverse templates are designed so that there is a stabilizing region at the 5' end, a nicking site downstream of the stabilizing region, and a recognition region downstream of the nicking site on the 3' end of the oligonucleotide. The total oligo length can range from 19 to 40, for example from 19-40, 23-40, 20-24, 23-24, 23-32, 25-40, 27-40, or 27-35 nucleotides depending on the length of each individual region, the temperature, the length of the target sequence, and the GC concentration. The templates may be designed so that they, together, would bind to less than or equal to 100% of the target sequence, one binding to the sense strand, and one to the antisense strand. For example, where the forward template binds to about 60% of the target antisense strand, the reverse template may, for example, bind to about 40% of the target sense strand. The templates may be designed to allow for spacer bases on the target sequence, that do not bind to either template. The templates thus may be designed to bind to about 30%, about 40%, about 50%, or about 60% of the target sequence.

The recognition region of the forward template is designed to be identical to the 5' region of the target sense strand and complementary to the 3' end of the target site antisense strand, for example, 8-16, 9-16, 10-16, 10-15, or 11-14 nucleotides long. In exemplary embodiments, the length is 12-13 nucleotides. The recognition region of the reverse template is designed to be complementary to the 3' end of the target site sense strand, for example, 8-16, 9-16, 10-16, 1015, or 11-14 nucleotides long. In exemplary embodiments, the length is 12-13 nucleotides.

In certain embodiments, the lengths of the recognition regions are adjusted so that there is at least one nucleotide in the target sequence that is not in the forward template's recognition region and also does not have its complement in the reverse template's recognition region. These spacer bases are nucleotides contained within the target sequence that lie in between the 3' ends of the forward and reverse templates. In certain embodiments, 5 spacer bases or less are present in the target sequence. In exemplary embodiments, the number of spacer bases is 2 to 3. In certain embodiments, the number of spacer bases is 1, 2, 3, 4, or 5. These spacer bases allow for

distinction of the true amplified product from any background products amplified by extension due to overlapping templates in a similar manner to primer-dimers. This consideration allows for improved discrimination between background and amplification of true target. However, these spacer bases are not required for the amplification to proceed.

The nicking site sequence of the template depends on which nicking enzyme is chosen for each template. Different nicking enzymes may be used in a single assay, but a simple amplification may, for example, employ a single nicking enzyme for use with both templates. Thus, the embodiments of the present invention include those where both templates comprise recognition sites for the same nicking enzyme, and only one nicking enzyme is used in the reaction. In these embodiments, both the first and second nicking enzymes are the same. The present invention also includes those embodiments where each template comprises a recognition site for a different nicking enzyme, and two nicking enzymes are used in the reaction.

For example, in the case of Nt.BstNBI, the enzyme binding site is 5'-GAGTC-3' and the enzyme nicks the top strand four nucleotides down stream of this site (i.e., GAGTCNNNN^). The amplification reaction shows little dependence on the sequence of these four nucleotides (N), though optimal sequence of this region is 25% or less GC content and with a thymine adjacent to the 5' nucleotide of the binding region. The latter stipulation allows for the priming ability of products that have an additional adenine added on by the polymerase. The sequence of the four nucleotides can be optimized to create or eliminate the presence of hairpins, self-dimers, or heterodimers, depending on the application.

The stabilizing region on the 5' end of the template oligonucleotide is designed to be roughly 50% GC. Thus, the GC content may be, for example, about 40%-60%, about 42%-58%, about 44%-56%, about 46%-54%, about 48%-52%, or about 49%-51%. These parameters result in a stabilizing region length of 8-11 nucleotides for the Nt.BstNBI enzyme, though lengths as short as 6 and as long as 15 nucleotides have been tested and were shown to work in this amplification method. Longer stabilizing regions or increased %GC to greater than 50% could further stabilize the nicking and extension reactions at higher reaction temperatures. The sequence of the 5' stabilizing regions of forward and reverse templates are usually identical, but can be varied if the aim is to capture each product strand independently. The sequence of this region should not interfere with the nicking site or the recognition region, though short internal

hairpins within the template sequence have been shown to have improved real-time results.

The templates of the present invention may include, for example, spacers, blocking groups, and modified nucleotides. Modified nucleotides are nucleotides or nucleotide triphosphates that differ in composition and/or structure from natural nucleotide and nucleotide triphosphates. Modified nucleotide or nucleotide triphosphates used herein may, for example, be modified in such a way that, when the modifications are present on one strand of a double-stranded nucleic acid where there is a restriction endonuclease recognition site, the modified nucleotide or nucleotide triphosphates protect the modified strand against cleavage by restriction enzymes. Thus, the presence of the modified nucleotides or nucleotide triphosphates encourages the nicking rather than the cleavage of the double-stranded nucleic acid. Blocking groups are chemical moieties that can be added to the template to inhibit target sequence-independent nucleic acid polymerization by the polymerase. Blocking groups are usually located at the 3' end of the template. Examples of blocking groups include, for example, alkyl groups, non-nucleotide linkers, phosphorothioate, alkane-diol residues, peptide nucleic acid, and nucleotide derivatives lacking a 3'-OH, including, for example, cordycepin. Examples of spacers, include, for example, C3 spacers. Spacers may be used, for example, within the template, and also, for example, at the 5' end, to attach other groups, such as, for example, labels.

Detailed Mechanism of Amplification

NEAR amplification requires the presence of a nucleic acid target, at least two template oligonucleotides, a thermophilic nicking enzyme, a thermophilic polymerase, and buffer components all held at the reaction temperature. The recognition region of the templates interacts with the complementary target sequence. Since the melting temperature of the complementary regions of the target and template is well below the reaction temperature, the interaction between the two nucleic acid strands is transient, but allows enough time for a thermophilic polymerase to extend from the 3' end of the template along the target strand. Experiments have shown that certain polymerases bind to single-stranded oligonucleotides. The pre-formation of this complex can facilitate the speed of the amplification process.

For a double-stranded target, both templates can interact with the corresponding target strands simultaneously (forward template with the antisense strand and reverse template with the

sense strand) during the normal breathing of double-stranded DNA. The target may also be generated by a single or double nick sites within the genome sequence. For a single-stranded target (either RNA or DNA), the reverse template binds and extends first (Figure 1, Step 1 and 2). The extended sequence contains the complement to the forward template. The forward template then displaces a region of the target and binds to the 3' synthesized region complementary to the recognition region of the forward template (Step 3). Alternatively, another reverse template can also displace the initial extended reverse template at the recognition region to create a single-stranded extended reverse template for the forward template to bind. The initial binding and extension of the templates is facilitated by a non-processive polymerase that extends shorter strands of DNA so that the melting temperature of the synthesized product is at or near the reaction temperature; therefore, a percentage of the product becomes single-stranded once the polymerase dissociates. The single-stranded product is then available for the next template recognition site to bind and polymerase to extend.

The forward template is extended to the *5'* end of the reverse template, creating a double-stranded nicking enzyme binding site for the reverse template (Step 5). The nicking enzyme then binds to the duplex and nicks directly upstream of the recognition sequence of the reverse template strand (in the case of a top-strand nicking enzyme) (Step 6). The nucleic acid sequence downstream of the nick is either released (if the melting temperature is near the reaction temperature) and/or is displaced by the polymerase synthesis from the *3-OH* nick site.

Polymerase extends along the forward template to the *5'* end of the forward template (Step 8).

The double-strand formed from the extension of both templates creates a nicking enzyme binding site on either end of the duplex. This double-strand is termed the NEAR amplification duplex. When nicking enzyme binds and nicks, either the target product located in between the two nick sites (with 5'-phosphate and 3-OH) is released, usually ranging in length from (but is

not limited to) 23 to 29 bases (Steps 9-11A), or the singly-nicked product containing the target product and the reverse complement of the nick site and stability region of the template (usually 36 to 48 bases in length) is released (Steps 9-11B). The ratio of products 1 to 2 can be adjusted by varying the concentrations of the templates. The forward:reverse template ratio may vary from, for example, molar ratios of 100:1, 75:1; 50:1, 40:1, 30:1, 20:1, 10:1, 5:1, 2.5:1, 1:1,

1:2.5, 1:5, 1:10, 1:20, 1:30, 1:40, 1:50, 1:75, or 1:100. The ratio of products (A to B) is dependent on the ratio of nicking enzyme to polymerase, *i.e.* a higher concentration of polymerase results in more of the longer length product (B) since there is comparatively less nicking enzyme to nick both strands simultaneously before the polymerase extends. Since displaced/released product of the reverse template feeds into the forward template and vice versa, exponential amplification is achieved. The nicking enzyme:polymerase ratio may vary from, for example, enzyme unit ratios of 20:1, 15:1; 10:1, 5:1, 4:1, 3:1, 2:1, 1.5:1, 1:1, 1:1.5, 1:2, 1:3, 1:4, 1:5, 1:10, 1:15, 1:20. In certain embodiments, the ratio of nicking enzyme to polymerase may, for example, be 1:3, 1:2, 1:1.5, or 1:0.8. Those of ordinary skill in the art recognize that these ratios may represent rounded values. This nicking and polymerase extension process continues until one of the resources (usually dNTPs or enzyme) is exhausted.

The time that the reaction is run may vary from, for example, 1-20 minutes, or 1-10, 1-8 , 1-5, 1-2.5, 2.5-5, 2.5-8, 2.5-10, or 2.5-20 minutes.

The methods of the present invention do not require the use of temperature cycling, as often is required in methods of amplification to dissociate the target sequence from the amplified nucleic acid. The temperature of the reaction may vary based on the length of the sequence, and the GC concentration, but, as understood by those of ordinary skill in the art, the temperature should be high enough to minimize non-specific binding. The temperature should also be suitable for the enzymes of the reaction, the nicking enzyme and the polymerase. For example, the reaction may be run at 37°C-85°C, 37°C-60°C, 54°C-60°C, and, in exemplary embodiments, from 55°C-59°C.

The polymerase may be mixed with the target nucleic acid molecule before, after, or at 15 the same time as, the nicking enzyme. In exemplary embodiments, a reaction buffer is optimized to be suitable for both the nicking enzyme and the polymerase.

Reactions may be allowed to completion, that is, when one of the resources is exhausted. Or, the reaction may be stopped using methods known to those of ordinary skill in the art, such as, for example, heat denaturation, or the addition of EDTA, high salts, or detergents. In exemplary embodiments, where mass spectrometry is to be used following amplification, EDTA may be used to stop the reaction.

Reaction Components

In a 1.5 mL Eppendorf tube combine the following reagents in order from top to bottom:

Reagent Added:	μL Per Reaction
H_2O	31.4
10X Thermopol Buffer (NEB)	5
10X NEB Buffer 3	2.5
100 mM $MgSO_4$	4.5
10 mM dNTPs	1.5
8 U/μl Bst Pol	0.6
10 U/μl N.BstNBI	1.5
20 μM Forward Template	0.25
20 μM Reverse Template	0.25
Total reaction mixture	47.5
Target sample	2.5
Total Reaction Volume	50 μL

The concentrations of components for the reaction conditions in this example are as follows:

Concentration	**Component**
45.7mM	Tris-HGI
13.9 mM	KCl
10 mM	$(NH_4)_2SO_2$
50 mM	NaCl
0.5mM	DTT
15 mM	$MgCl_2$
0.10%	Triton X-100
0.008 mM	EDTA
6 μg/mL	BSA
3.90%	Glycerol (can be lower if using more concentrated enzyme stock)
0.3 U/μL	NT.BstNBI
0.1-0.4 U/μL	Bst polymerase (large fragment)
0.1 μM	Forward template
0.1 μM	Reverse template

Variations in buffer conditions, $MgSO_4$ concentration, polymerase concentration, and template concentrations all can be optimized based on the assay sequence and desired detection

method. The amount of glycerol may, for example, be lowered if a more concentrated enzyme stock is used. Also, those of ordinary skill in the art recognize that the reaction may be run without EDTA or BSA; these components may be present in the reaction as part of the storage buffers for the enzymes. The volumes can be scaled for larger or smaller total reaction volumes. The volume is usually between 5 µL and 100 µL.

The template concentrations are typically in excess of the concentration of target. The concentrations of the forward and reverse templates can be at the same or at different concentrations to bias the amplification of one product over the other. The concentration of each is usually between 10 nM and 1µM.

Additives such as BSA, non-ionic detergents such as Triton X-100 or Tween-20, DMSO, DTT, and RNase inhibitor may be included for optimization purposes without adversely affecting the amplification reaction.

Preparing/Adding Target

Targets may be diluted in 1 x Thermopol Buffer II, 1 x TE (pH 7.5) or H_20. Hot start conditions allow for faster, more specific amplification. In this case, the reaction mix (minus either enzymes or templates and target) is heated to the reaction temperature for 2 minutes, after which the reaction mix is added to the other component (enzymes or templates/target). The target can be added in any volume up to the total amount of water required in the reaction. In this case, the target would be diluted in water. In the example above for a 50 µL total reaction volume, 2.5 µL of the prepared target should be added per reaction to bring the total reaction volume to 50 µL.

Running the Reaction

The reaction is run at a constant temperature, usually between 54°C and 60°C for the enzyme combination of Bst polymerase (large fragment) and Nt.Bst.NB 1 nicking enzyme. Other enzyme combinations may be used and the optimal reaction temperature will be based on the optimal temperature for both the nicking enzyme and polymerase to work in concert as well as the melting temperature of the reaction products. The reaction is held at temperature for 2.5 to 10 minutes until the desired amount of amplification is achieved. The reaction may be stopped by

either a heat denaturation step to denature the enzymes (when using enzymes that can be heat-killed). Alternatively, the reaction may be stopped by adding EDTA to the reaction.

Readout

The amplified target sequence may be detected by any method known to one of ordinary skill in the art. By way of non-limiting example, several of these known methods are presented herein. In one method, amplified products may be detected by gel electrophoresis, thus detecting reaction products having a specific length. The nucleotides may, for example, be labeled, such as, for example, with biotin. Biotin-labeled amplified sequences may be captured using avidin bound to a signal generating enzyme, for example, peroxidase.

Nucleic acid detection methods may employ the use of dyes that specifically stain double-stranded DNA. Intercalating dyes that exhibit enhanced fluorescence upon binding to DNA or RNA are a basic tool in molecular and cell biology. Dyes may be, for example, DNA or RNA intercalating fluorophores and may include but are not limited to the following examples: Acridine orange, ethidium bromide, Hoechst dyes, PicoGreen, propidium iodide, SYBR I (an asymmetrical cyanine dye), SYBR II, TOTO (a thiaxole orange dimer) and YOYO (an oxazole yellow dimer). Dyes provide an opportunity for increasing the sensitivity of nucleic acid detection when used in conjunction with various detection methods and may have varying optimal usage parameters. For example ethidium bromide is commonly used to stain DNA in agarose gels after gel electrophoresis and during peR (Hiquchi et al., Nature Biotechnology 10; 413-417, April 1992), propidium iodide and Hoechst 33258 are used in flow cytometry to determine DNA ploidy of cells, SYBR Green 1 has been used in the analysis of double-stranded DNA by capillary electrophoresis with laser induced fluorescence detection and Pico Green has been used to enhance the detection of double-stranded DNA after matched ion pair polynucleotide chromatography (Singer et al., Analytical Biochemistry 249,229-238 1997).

Nucleic acid detection methods may also employ the use of labeled nucleotides incorporated directly into the target sequence or into probes containing complementary sequences to the target of interested. Such labels may be radioactive and/or fluorescent in nature and can be resolved in any of the manners discussed herein.

Methods of detecting and/or continuously monitoring the amplification of nucleic acid

products are also well known to those skilled in the art and several examples are described below.

The production or presence of target nucleic acids and nucleic acid sequences may be detected and monitored by Molecular Beacons. Molecular Beacons are hair-pin shaped oligonucleotides containing a fluorophore on one end and a quenching dye on the opposite end. The loop of the hair-pin contains a probe sequence that is complementary to a target sequence and the stem is formed by annealing of complementary arm sequences located on either side of the probe sequence. A fluorophore and a quenching molecule are covalently linked at opposite ends of each arm. Under conditions that prevent the oligonucleotides from hybridizing to its complementary target or when the molecular beacon is free in solution the fluorescent and quenching molecules are proximal to one another preventing fluorescence resonance energy transfer (FRET). When the molecular beacon encounters a target molecule, hybridization occurs; the loop structure is converted to a stable more rigid conformation causing separation of the fluorophore and quencher molecules leading to fluorescence (Tyagi et al. Nature Biotechnology 14: March 1996,303-308). Due to the specificity of the probe, the generation of fluorescence is exclusively due to the synthesis of the intended amplified product.

Molecular beacons are extraordinarily specific and can discern a single nucleotide polymorphism. Molecular beacons can also be synthesized with different colored fluorophores and different target sequences, enabling several products in the same reaction to be quantitated simultaneously. For quantitative amplification processes, molecular beacons can specifically bind to the amplified target following each cycle of amplification, and because non-hybridized molecular beacons are dark, it is not necessary to isolate the probe-target hybrids to quantitatively determine the amount of amplified product. The resulting signal is proportional to the amount of amplified product. This can be done in real time. As with other real time formats, the specific reaction conditions must be optimized for each primer/probe set to ensure accuracy and precision.

The production or presence of target nucleic acids and nucleic acid sequences may also be detected and monitored by Fluorescence resonance energy transfer (FRET). FRET is an energy transfer mechanism between two chromophores: a donor and an acceptor molecule. Briefly, a donor fluorophore molecule is excited at a specific excitation wavelength. The

subsequent emission from the donor molecule as it returns to its ground state may transfer excitation energy to the acceptor molecule through a long range dipole-dipole interaction. The intensity of the emission of the acceptor molecule can be monitored and is a function of the distance between the donor and the acceptor, the overlap of the donor emission spectrum and the acceptor absorption spectrum and the orientation of the donor emission dipole moment and the acceptor absorption dipole moment. FRET is a useful tool to quantify molecular dynamics, for example, in DNA-DNA interactions as seen with Molecular Beacons. For monitoring the production of a specific product a probe can be labeled with a donor molecule on one end and an acceptor molecule on the other. Probe-target hybridization brings a change in the distance or orientation of the donor and acceptor and FRET change is observed. (Joseph R. Lakowicz, “Principles of Fluorescence Spectroscopy”, Plenum Publishing Corporation, 2nd edition (July 1, 1999)).

The production or presence of target nucleic acids and nucleic acid sequences may also be detected and monitored by Mass Spectrometry. Mass Spectrometry is an analytical technique that may be used to determine the structure and quantity of the target nucleic acid species and can be used to provide rapid analysis of complex mixtures. Following the method, samples are ionized, the resulting ions separated in electric and/or magnetic fields according to their mass-to-charge ratio, and a detector measures the mass-to-charge ratio of ions. (Crain, P. F. and McCloskey, J. A., Current Opinion in Biotechnology 9: 25-34 (1998)). Mass spectrometry methods include, for example, MALDI, MALDIITOF, or Electrospray. These methods may be combined with gas chromatography (GC/MS) and liquid chromatography (LC/MS). MS has been applied to the sequence determination of DNA and RNA oligonucleotides (Limbach P., MassSpectrom. Rev. 15: 297-336 (1996); Murray K., J. Mass Spectrom. 31: 1203-1215 (1996)). MS and more particularly, matrix-assisted laser desorption/ionization MS (MALDI MS) has the potential of very high throughput due to high-speed signal acquisition and automated analysis off solid surfaces. It has been pointed out that MS, in addition to saving time, measures an intrinsic property of the molecules, and therefore yields a significantly more informative signal (Koster H. et al., Nature Biotechnol., 14: 1123-1128 (1996)).

The production or presence of target nucleic acids and nucleic acid sequences may also be detected and monitored by various methods of gel electrophoresis. Gel electrophoresis

involves the separation of nucleic acids through a matrix, generally a cross-linked polymer, using an electromotive force that pulls the molecules through the matrix. Molecules move through the matrix at different rates causing a separation between products that can be visualized and interpreted via anyone of a number of methods including but not limited to; autoradiography, phosphorimaging, and staining with nucleic acid chelating dyes.

The production or presence of target nucleic acids and nucleic acid sequences may also be detected and monitored by capillary gel electrophoresis. Capillary-gel Electrophoresis (CGE) is a combination of traditional gel electrophoresis and liquid chromatography that employs a medium such as polyacrylamide in a narrow bore capillary to generate fast, high-efficient separations of nucleic acid molecules with up to single base resolution. CGE is commonly combined with laser induced fluorescence (LIF) detection where as few as six molecules of stained DNA can be detected. CGE/LIF detection generally involves the use of fluorescent DNA intercalating dyes including ethidium bromide, YOYO and SYBR Green 1 but can also involve the use of fluorescent DNA derivatives where the fluorescent dye is covalently bound to the DNA. Simultaneous identification of several different target sequences can be made using this method.

The production or presence of target nucleic acids and nucleic acid sequences may also be detected and monitored by various surface capture methods. This is accomplished by the immobilization of specific oligonucleotides to a surface producing a biosensor that is both highly sensitive and selective. Surfaces used in this method may include but are not limited to gold and carbon and may use a number of covalent or noncovalent coupling methods to attach the probe to the surface. The subsequent detection of a target DNA can be monitored by a variety of methods.

Electrochemical methods generally involve measuring the cathodic peak of intercalators, such as methylene blue, on the DNA probe electrode and visualized with square wave voltammograms. Binding of the target sequence can be observed by a decrease in the magnitude of the voltammetric reduction signals of methylene blue as it interacts with dsDNA and ssDNA differently reflecting the extent of the hybrid formation.

Surface Plasmon Resonance (SPR) can also be used to monitor the kinetics of probe attachment as well as the process of target capture. SPR does not require the use of fluorescence probes or other labels. SPR relies on the principle of light being reflected and refracted on an

interface of two transparent media of different refractive indexes. Using monochromatic and p-polarized light and two transparent media with an interface comprising a thin layer of gold, total reflection of light is observed beyond a critical angle, however the electromagnetic field component of the light penetrates into the medium of lower refractive index creating an evanescent wave and a sharp shadow (surface plasmon resonance). This is due to the resonance energy transfer between the wave and the surface plasmons. The resonance conditions are influenced by the material absorbed on the thin metal film and nucleic acid molecules, proteins and sugars concentrations are able to be measured based on the relation between resonance units and mass concentration.

The production or presence of target nucleic acids and nucleic acid sequences may also be detected and monitored by lateral flow devices. Lateral Flow devices are well known. These devices generally include a solid phase fluid permeable flow path through which fluid flows through by capillary force. Examples include, but are not limited to, dipstick assays and thin layer chromatographic plates with various appropriate coatings. Immobilized on the flow path are various binding reagents for the sample, binding partners or conjugates involving binding partners for the sample and signal producing systems. Detection of samples can be achieved in several manners; enzymatic detection, nanoparticle detection, colorimetric detection, and fluorescence detection, for example. Enzymatic detection may involve enzyme-labeled probes that are hybridized to complementary nucleic aid targets on the surface of the lateral flow device. The resulting complex can be treated with appropriate markers to develop a readable signal. Nanoparticle detection involves bead technology that may use colloidal gold, latex and paramagnetic nanoparticles. In one example, beads may be conjugated to an anti-biotin antibody. Target sequences may be directly biotinylated, or target sequences may be hybridized to a sequence specific biotinylated probes. Gold and latex give rise to colorimetric signals visible to the naked eye and paramagnetic particles give rise to a non-visual signal when excited in a magnetic field and can be interpreted by a specialized reader.

Fluorescence-based lateral flow detection methods are also known, for example, dual fluorescein and biotin-labeled oligo probe methods, UPT -N ALP utilizing up-converting phosphor reporters composed of lanthanide elements embedded in a crystal (Corstjens et al., Clinical Chemistry, 47:10,1885-1893,2001), as well as the use of quantum dots.

Nucleic acids can also be captured on lateral flow devices. Means of capture may include antibody dependent and antibody independent methods. Antibody-dependent capture generally comprises an antibody capture line and a labeled probe of complementary sequence to the target. Antibody-independent capture generally uses non-covalent interactions between two binding partners, for example, the high affinity and irreversible linkage between a biotinylated probe and a streptavidin line. Capture probes may be immobilized directly on lateral flow membranes. Both antibody dependent and antibody independent methods may be used in multiplexing.

The production or presence of target nucleic acids and nucleic acid sequences may also be detected and monitored by multiplex DNA sequencing. Multiplex DNA sequencing is a means of identifying target DNA sequences from a pool of DNA. The technique allows for the simultaneous processing of many sequencing templates. Pooled multiple templates can be resolved into individual sequences at the completion of processing. Briefly, DNA molecules are pooled, amplified and chemically fragmented. Products are fractionated by size on sequencing gels and transferred to nylon membranes. The membranes are probed and autoradiographed using methods similar to those used in standard DNA sequencing techniques (Church et al., Science 1998 Apr 8;240(4849):185-188). Autoradiographs can be evaluated and the presence of target nucleic acid sequence can be quantitated.

Kits

Kits of the present invention may comprise, for example, one or more polymerases, forward and reverse templates, and one or more nicking enzymes, as described herein. Where one target is to be amplified, one or two nicking enzymes may be included in the kit. Where multiple target sequences are to be amplified, and the templates designed for those target sequences comprise the nicking enzyme sites for the same nicking enzyme, then one or two nicking enzymes may be included. Or, where the templates are recognized by different nicking enzymes, more nicking enzymes may be included in the kit, such as, for example, 3 or more.

The kits of the present invention may also comprise one or more of the components in any number of separate containers, packets, tubes, vials, microtiter plates and the like, or the components may be combined in various combinations in such containers.

The components of the kit may, for example, be present in one or more containers, for

example, all of the components may be in one container, or, for example, the enzymes may be in a separate container from the templates. The components may, for example, be lyophilized, freeze dried, or in a stable buffer. In one example, the polymerase and nicking enzymes are in lyophilized form in a single container, and the templates are either lyophilized, freeze dried, or in buffer, in a different container. Or, in another example, the polymerase, nicking enzymes, and the templates are, in lyophilized form, in a single container. Or, the polymerase and the nicking enzyme may be separated into different containers.

Kits may further comprise, for example, dNTPs used in the reaction, or modified nucleotides, cuvettes or other containers used for the reaction, or a vial of water or buffer for re-hydrating lyophilized components. The buffer used may, for example, be appropriate for both polymerase and nicking enzyme activity.

The kits of the present invention may also comprise instructions for performing one or more methods described herein and/or a description of one or more compositions or reagents described herein. Instructions and/or descriptions may be in printed form and may be included in a kit insert. A kit also may include a written description of an Internet location that provides such instructions or descriptions.

Kits may further comprise reagents used for detection methods, such as, for example, reagents used for FRET, lateral flow devices, dipsticks, fluorescent dye, colloidal gold particles, latex particles, a molecular beacon, or polystyrene beads.

Examples

Example 1: Detection of DNA NEAR assay products by gel electrophoresis

The NEAR amplification reaction products can be visualized by gel electrophoresis. In the absence of target, the templates (with complementary 3' bases) overlap by one or more bases, polymerase extends in each direction to generate the NEAR amplification duplex (Figure IB); and the amplification proceeds in a similar mechanism to the NEAR amplification to amplify a product that is two bases shorter than the target amplified product. In the case of a 25mer assay where the templates end in A and T, the resulting background product is 23 bases. The 27mer assay also forms a 23mer background and 27mer product. Longer reaction products are also amplified. The sequence of these products is hypothesized to be due to the polymerase extension

before the nicking enzyme can nick both sides of the NEAR amplification duplex, according to Steps 9B in Figure Ie. Figure 2 shows the NEAR reaction products are easily distinguished from background products by gel electrophoresis.

Example 2: Detection of RNA NEAR assay products by gel electrophoresis

The NEAR reaction can also amplify RNA targets. In this case, the target is Ebola Armored RNA, which is a -600 base strand of RNA encapsulated by MS2 phage coat proteins to simulate a viral particle. The reaction is designed to amplify a 25-base region of the Ebola genome contained within the encapsulated RNA sequence. Reaction products run on a 20% polyacrylamide gel (Figure 3) show the amplified 25mer product along with 23mer and 20mer background products. This example demonstrates the ability of the NEAR reaction to amplify RNA released from virus-like particles.

Example 3: Detection of DNA and RNA NEAR assay products by mass spectrometry

The NEAR reaction amplification products can also be detected by mass spectrometry using an ESI/TOF system with a front end LC. The reaction products observed are multiple charged ion species. Usually, the -3 or -4 charge state is the major peak in the spectrum (in the range of 1000-3000 AMU), depending on the length of the oligonucleotide product. The sodium adduct is usually present in the spectrum as a peak adjacent to the major peak at roughly 20-25% the intensity. The unique peaks for the positive reactions in the presence of target are visible in both Figures 4 and 5 for the DNA and RNA NEAR reactions respectively. The background products formed in these NEAR reactions are not shown in the mass range of these spectra.

Example 4: Real-time detection of the NEAR assay amplification

The NEAR amplification reaction can also be monitored, as shown in Figure 6, in real-time with SYBR II fluorescence. The fluorescence increases as SYBR II intercalates into the amplified double-stranded products. The background products also generate fluorescence at a slower rate than the true product. Optimization of amplification sequence, reaction temperature and reaction buffer conditions are necessary in order to visualize distinct separation between the positive reactions and the negative controls.

Example 5: FRET detection of real-time NEAR assay amplification

NEAR amplification can also be monitored by Fluorescence Resonance Energy Transfer (FRET), as shown in Figure 7. Amplification occurs using dual labeled templates, one on each end (5-FAM, 3-BHQ). Fluorescence is generated from the FAM-labeled oligonucleotide upon cleavage of the template by the nicking enzyme when it becomes double-stranded. Since fluorescence is produced by the initial nicking reaction, this detection method is extremely responsive. Since the 3' ends of the templates are blocked from extension by the quenching label, the production of background fluorescence is inhibited.

Example 6: Molecular beacon detection of real-time NEAR amplification

A third method of monitoring real-time amplification is using molecular beacons, as shown in Figure 8. In this case, the amplified product hybridizes to the loop region of the molecular beacon resulting in an increase in fluorescence from the separation of the fluorophore and quencher on each end of the hairpin stem. Since this interaction occurs post-amplification, it is considered pseudo-real-time and can be slightly slower in response relative to the FRET approach.

Example 7: False Alarm Rate testing

This experiment was designed to probe the probability that the NEAR amplification reaction will yield a true product in the negative reaction, or a false positive. NEAR reactions directed at specific amplification of a 25mer region specific to the *Bacillus subtilis* genome were run in the presence (n = 120) and absence (n =320) of *Bacillus subtilis* genomic DNA. End point reactions were run on the mass spectrometer and the area under the curve (AUC) for the product mass peak in the mass spectrum was analyzed. As shown in Figure 9, the results show that none of the 320 negative reactions resulted in a false positive with AUC values equal to the water control. The true positive AUC values were at least 3 standard deviations apart from the true negatives. Overall, these results demonstrate the reproducible nature of the NEAR assay.

Example 8: Beacon detection: NEAR assay reproducibility with beacon detection

The molecular beacon detection of NEAR reaction products can also be used as an endpoint reading. As shown in Figure 10, the ratio of NEAR reaction products can be manipulated by varying the input ratio of the forward and reverse templates. Skewing the templates to favor one of the reaction products allows the single-stranded product to be available for hybridization to a molecular beacon. The open beacon generates a fluorescent signal. This detection method is extremely reproducible. In this study, two operators performed replicates of the same assay on two different days. The results of this study demonstrate the reproducibility of the assay from one day to the next as well as reproducibility between operators.

Example 9: NEAR Assay sensitivity with beacon detection

The sensitivity of the NEAR assay with beacon read-out was tested using a dilution of *Francisella tularensis* genomic DNA. As shown in Figure 11, as few as 50 copies were detected above the no target control.

Example 10: Concentration of amplified products for NEAR DNA amplification

The sensitivity of the NEAR assay has also been studied using mass spectrometry detection of the reaction products. Figure 12 shows signal above the no target control down to 100 copies. The data from this study was used to correlate the input copy number to the final amount of amplified product. In this study, the *AUC* values of the mass spec product peaks were fit to a standard curve to give the estimated final concentration of amplified product for the NEAR assay. The amount of amplified product ranges from approximately 250nM to almost lμM for 1E+2 and 1E+5 copies respectively. This product amount results in a 1E+8 to 7E+ 10-fold amplification. These reactions were performed without the hot-start conditions, in fact hot-start conditions have been shown to dramatically increase the amount of product amplified, so a further increase in amplification is achieved. The zero copy amplification reaction has a positive final concentration due to the y-intercept value in the standard curve equation.

Example 11: Concentration of amplified products for RNA assay

A similar study was performed on the NEAR amplification of RNA. A dilution of RNA targets were amplified by the NEAR assay. Products were run on the mass spec and the *AUC*

values of the product peaks were analyzed against a standard curve to determine the concentration of the final product, as shown in Figure 13. A 12 minute amplification starting with 30 and 30,000 copies of initial target results in a 3E+9 to 1E+7 -fold amplification respectively. The lower extent of amplification compared to the DNA amplification could be due to the less efficient reverse transcriptase ability of the polymerase compared to its replication abilities. Also, the RNA:DNA hybrid formed upon the extension of the reverse template is a stronger interaction compared to a normal DNA:DNA hybrid and will have less breathing to allow for the forward or another reverse template to displace one strand. However, amplification products from the RNA reaction were detected down to <100 copies.

Example 12: NEAR reaction specificity for DNA

Since the reaction products are usually between 20 and 30 bases in length, the question arises as to whether or not these short amplification assays can be specific enough to target a single sequence region with other near neighbor genomes present. The NEAR reaction was tested for its specificity by running the amplification reaction in the presence and absence of varying amounts of the near neighbor genomic DNA (Figure 14). In this case, the assay detects a specific sequence in the pX02 plasmid of *Bacillus anthracis* and the near neighbor genome is *Bacillus thuringiensis* (kurstaki). The reactions were analyzed by the AUC values for the product peaks. The figure below demonstrates that in the absence of the correct target *(Bacillus anthracis),* there is no true product amplified (the levels are so low that they are not visible on the scale of the graph). The amount of amplification of the positive reactions is consistent, with larger error bars for the 0 and 5E+5 copies of *Bacillus thuringiensis* (5E+4 copies of *Bacillus anthracis)* due to a single lower value for one of the triplicate runs. Overall the experiment demonstrates that the NEAR reaction is very specific to the target sequence when the assay is designed within a unique region of the genome.

Example 13: Interferent testing

A panel of interferents was tested to monitor the effect of each on the NEAR assay amplification. Figure 15 demonstrates the robust nature of the NEAR assay in the presence of interferents. Some interferents that are known to inhibit PCR, such as humic acid, did not appear

to inhibit the NEAR assay, though the amount of each interferent is unknown. From statistical analysis only interferent B, C, and E were statistically different from the control assay x. In the B, C, and E cases, the difference resulted in increased product amplification.

Example 14: Multiplexing of two sequences with NEAR DNA assays

A DNA duplex was designed for capillary electrophoresis (CE) detection. Amplification products were 25 bases *(Bacillus anthracis* assay, *Ba)* and 27 bases *(Bacillus subtilis* assay, *Bs)* in length with background production of a 23mer. The reaction was run for 10 minutes in the presence or absence of 5E+5 copies of the respective genomic DNA target. The samples were run on a 20% polyacrylamide gel to visualize the reaction products. Figure 16 indicates the presence of positive product amplification when *Bacillus subtilis* only is present as well as when both *Bacillus subtilis* and *Bacillus anthracis* are present.

Example 15: NEAR DNA assay duplex specificity

The NEAR DNA duplex reaction with *Bacillus subtilis (Bs)* and *Bacillus anthracis (Ba)* was shown to be specific to the respective genomes. The assays were run in the presence of the near neighbor, *Bacillus thuringiensis,* as shown in Figure 17. In the negative reaction where both template sets are present as well as the *Bacillus thuringiensis* genomic DNA, there is no product band in the 25 or 27mer region. Product bands appear only when the specific genomic target is present, which demonstrates the specificity of the duplex reaction.

Example 16: Multiplexing with NEAR RNA assays

An MS2 assay that amplifies a 27mer product and an Ebola assay that amplifies a 25mer product was developed and multiplexed so that all templates are present in each assay and amplification of products is dependent on the target present. This combination of templates forms background products that are 23 bases and 20 bases in length. The gel shown in Figure 18 demonstrates the ability for the NEAR reaction to amplify multiple RNA targets in a single reaction.

Example 17: Amplification from lysed spores by NEAR Assay

Amplification was performed on semi-processed samples to determine whether it is possible to amplify DNA released from spores through lysis. The negative control reaction contained DNase-treated spores, unlysed, so no DNA should be present to amplify. The positive control reaction contained purified genomic DNA at concentrations around the amount of DNA estimated to be released through lysis. Results in Figure 19 show that amplification with unlysed DNase-treated spores results in no product amplification as expected, whereas the three samples lysed before amplification resulted in product amounts in the range of the theoretical amounts.

Example 18: Capture and Extension

The NEAR reaction products can also be detected on a solid surface. A capture probe attached at the 5' end to the surface through a biotin/streptavidin attachment can bind to the reaction products from which polymerase extends to form a stable duplex that SYBR and any intercalating dye can detect. The capture probe is designed to favor extension through binding to the true product over background products because the 3' base of the capture probe is complementary to the middle spacer base in the product which is not present in either of the templates or the background products. Figure 20 demonstrates the increased fluorescence of the NEAR products in the presence of the capture probe and polymerase over the average binding (same reaction in the absence of polymerase, to preclude extension of the capture probe) and the no target control where only background products are amplified, but cannot form a stable duplex with the capture probe for polymerase to extend.

Example 19: Surface NEAR FRET DNA Assay

The NEAR reaction can also be performed with the templates immobilized on the surface. The templates for FRET detection of surface amplification usually have three modifications: one 5' biotin with a TEG spacer, one FAM fluorophore internal to the biotin, and a quencher on the 3' end which serves to block background amplification as well as to quench the FAM fluorophore. The template is immobilized on the surface through biotin/streptavidin attachment. Figure 21 demonstrates that with both templates immobilized along with additional mixing, the reaction proceeds at a much slower rate than the solution amplification rate (amplification in 16 minutes for lE+6 copies of genomic DNA). When a single template is

immobilized on the surface and the other template is free in solution, the amplification reaction is increased to 10 minute detection for lE+6 copies of genomic DNA. Fluorescence from background products is observed -3.5 minutes after the product signal, similar to what is observed for solution phase kinetics, but slowed considerably.

****

The entirety of each patent, patent application, publication and document referenced herein hereby is incorporated by reference. Citation of the above patents, patent applications, publications and documents is not an admission that any of the foregoing is pertinent prior art, nor does it constitute any admission as to the contents or date of these publications or documents.

Singular forms “a”, “an”, and “the” include plural reference unless the context clearly dictates otherwise. Thus, for example, reference to “a subset” includes a plurality of such subsets, reference to “a nucleic acid” includes one or more nucleic acids and equivalents thereof known to those skilled in the art, and so forth. The term “or” is not meant to be exclusive to one or the terms it designates. For example, as it is used in a phrase of the structure “A or B” may denote A alone, B alone, or both A and B.

Unless defined otherwise, all technical and scientific terms used herein have the same meanings as commonly understood by one of ordinary skill in the art to which this invention belongs. Although any methods and systems similar or equivalent to those described herein can be used in the practice or testing of the present invention, the methods, devices, and materials are now described. All publications mentioned herein are incorporated herein by reference for the purpose of describing and disclosing the processes, systems, and methodologies that are reported in the publications which might be used in connection with the invention. Nothing herein is to be construed as an admission that the invention is not entitled to antedate such disclosure by virtue of prior invention.

Modifications may be made to the foregoing without departing from the basic aspects of the invention. Although the invention has been described in substantial detail with reference to one or more specific embodiments, those of ordinary skill in the art will recognize that changes may be made to the embodiments specifically disclosed in this application, and yet these modifications and improvements are within the scope and spirit of the invention. The invention illustratively described herein suitably may be practiced in the absence of any element(s) not

specifically disclosed herein. Thus, for example, in each instance herein any of the terms "comprising", "consisting essentially of", and "consisting of" may be replaced with either of the other two terms. Thus, the terms and expressions which have been employed are used as terms of description and not of limitation, equivalents of the features shown and described, or portions thereof, are not excluded, and it is recognized that various modifications are possible within the scope of the invention. Embodiments of the invention are set forth in the following claims.

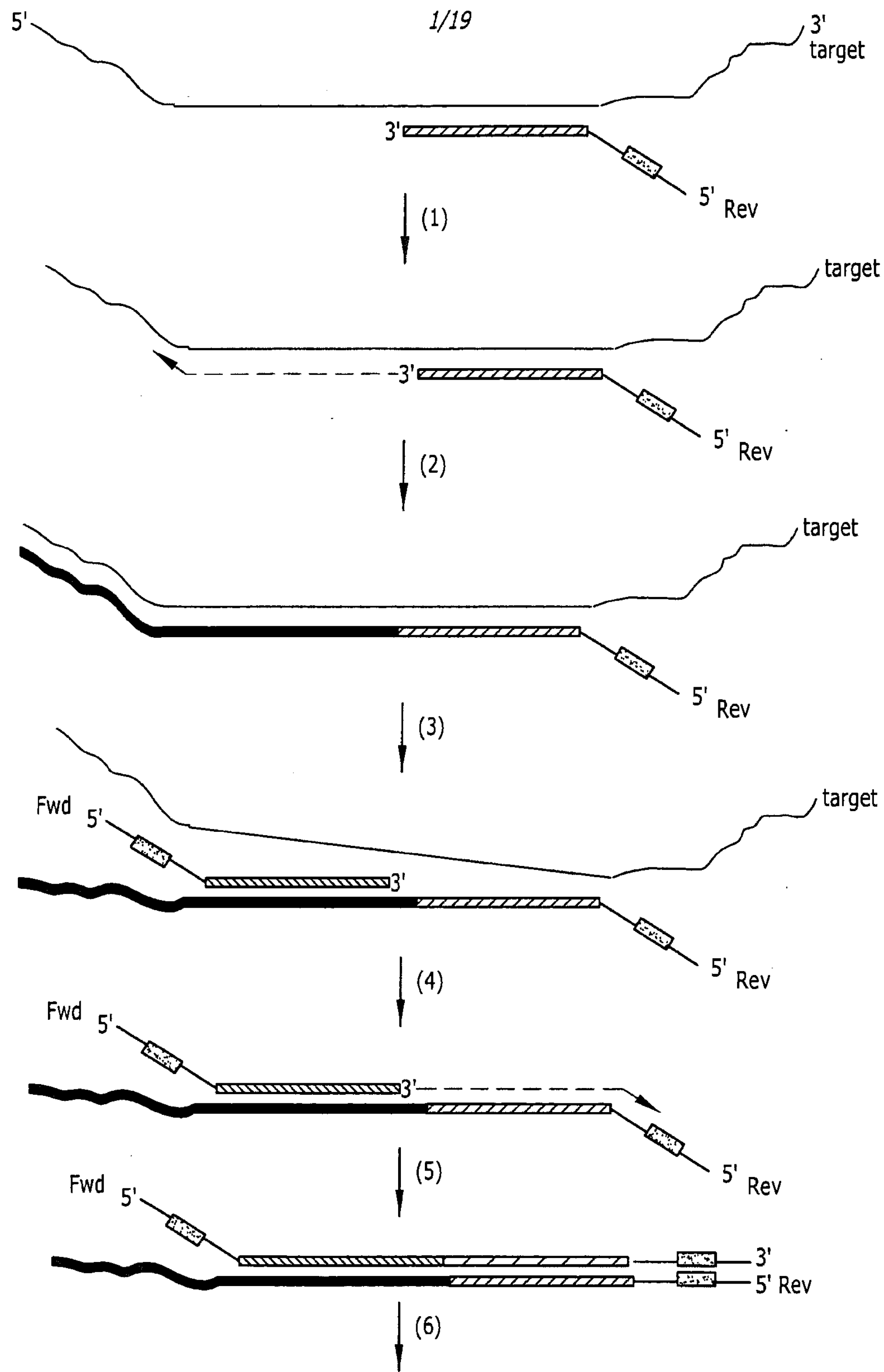


FIG. 1A

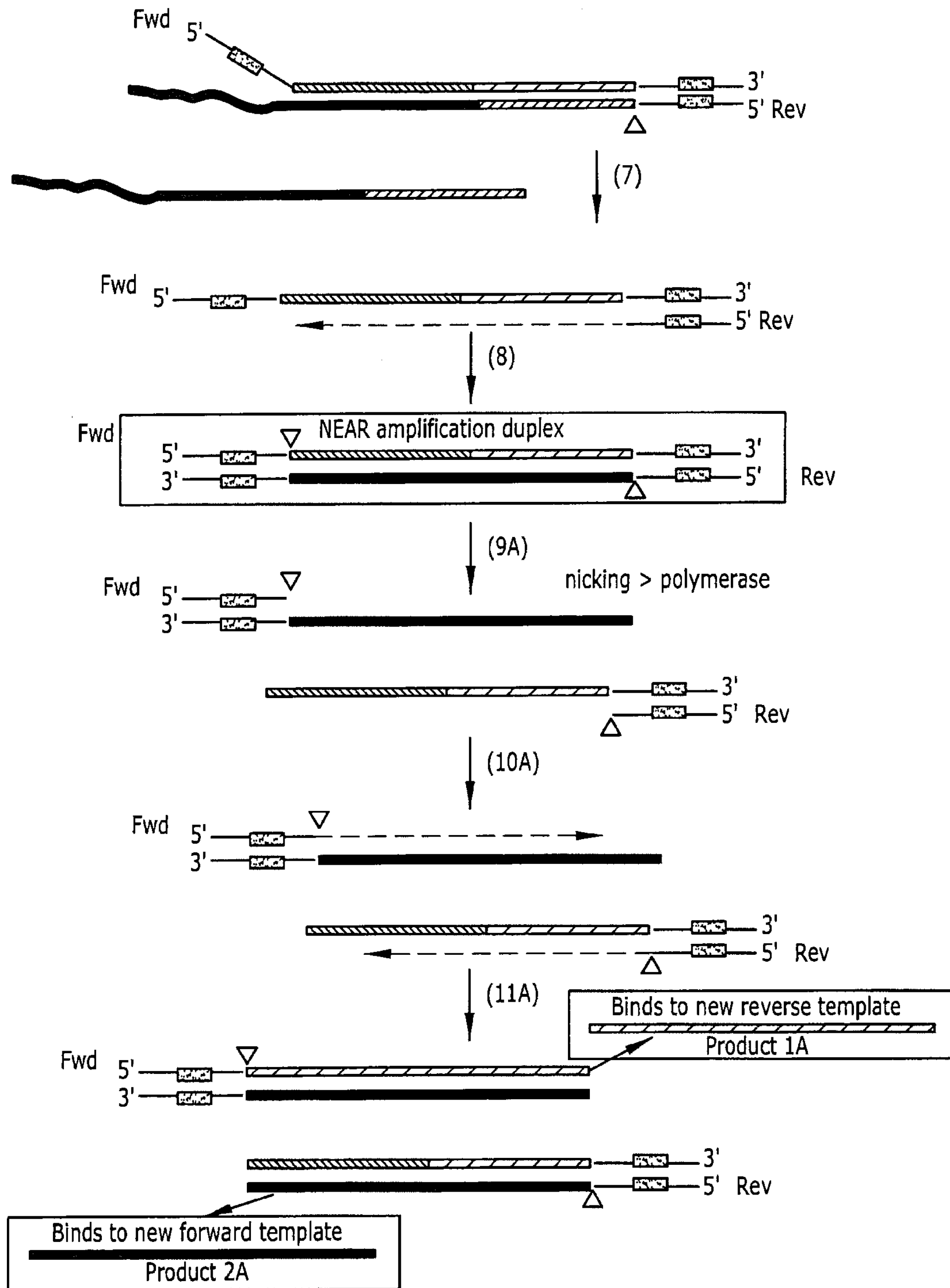


FIG. 1B

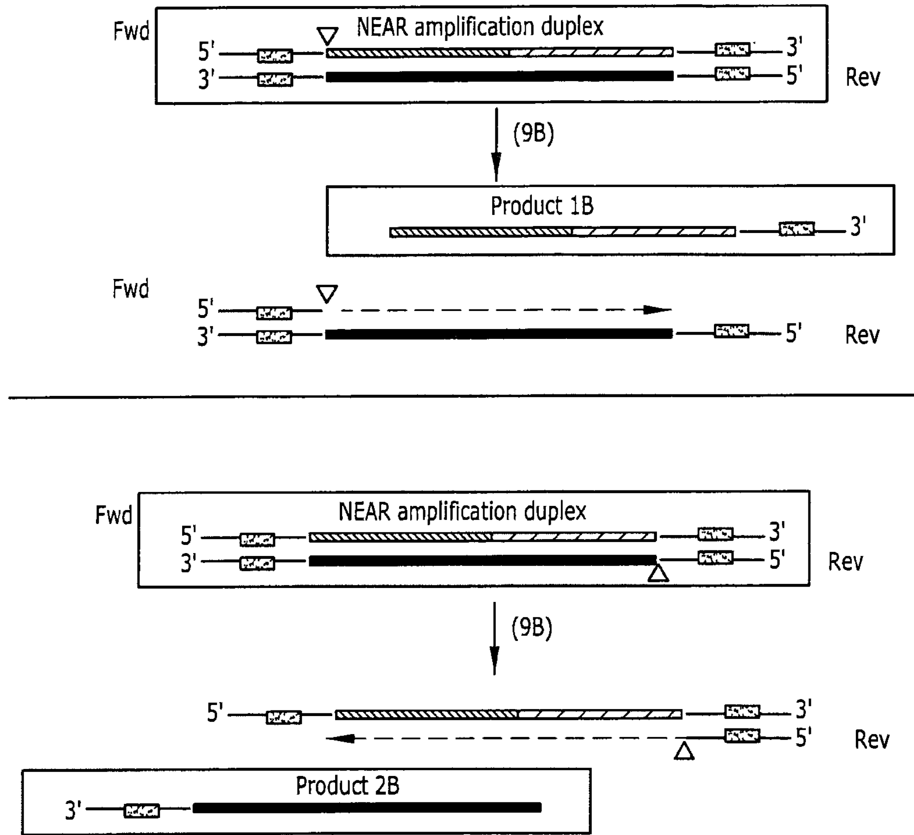


FIG. 1C

Recognition region to
sense strand of target

Nicking Site

Nicking Site

Recognition site to
antisense strand of target

FIG. 1D

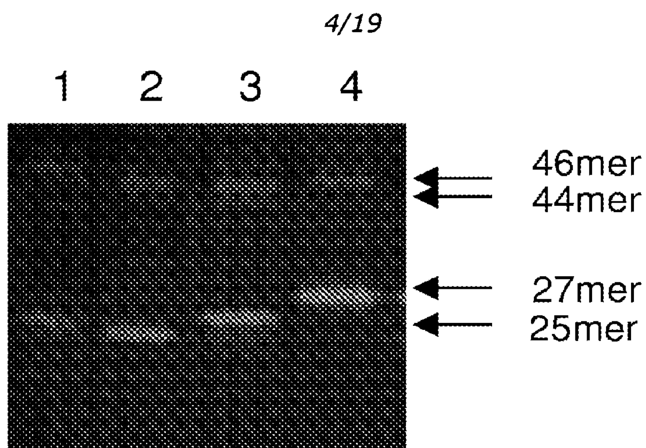


FIG. 2

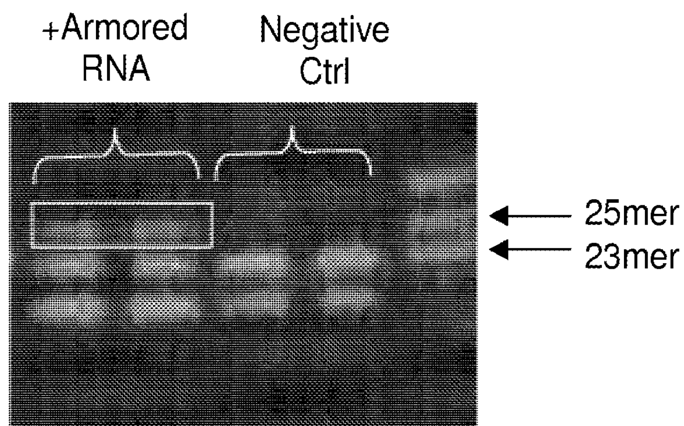
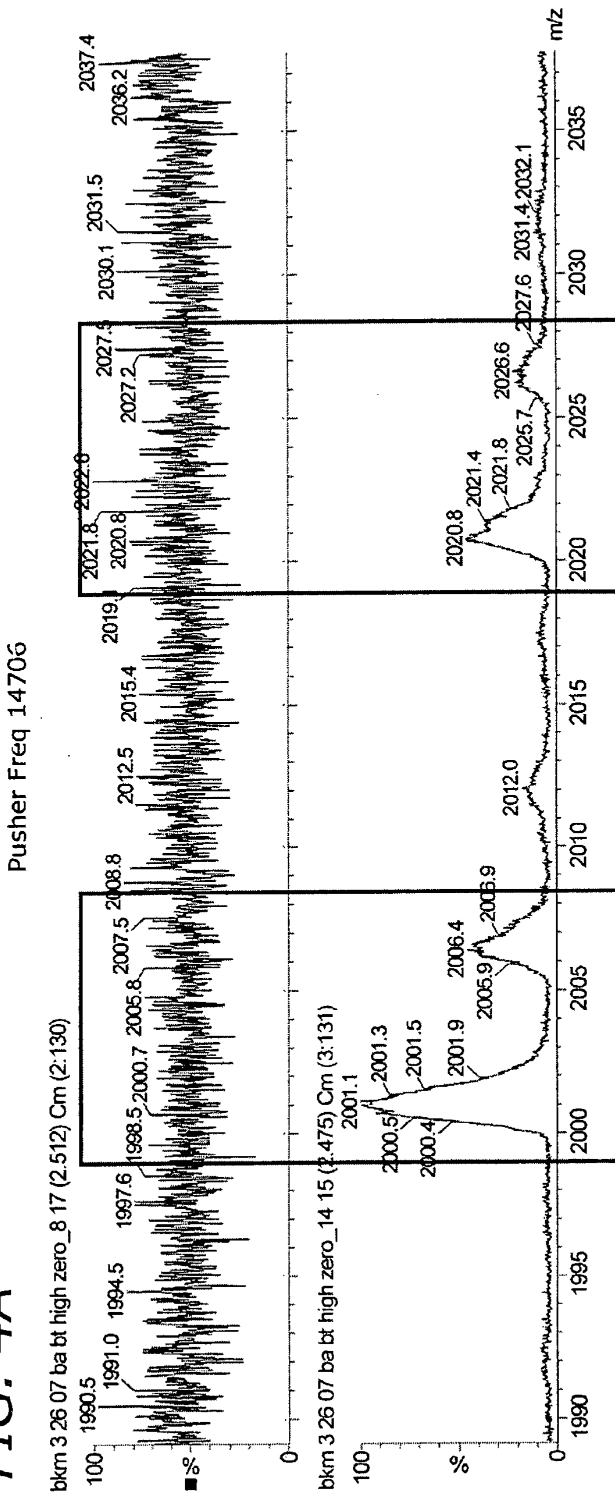


FIG. 3


Pusher Freq 14706
bkm 3 26 07 ba bt high zero_8 17 (2.512) Cm (2:130)
bkm 3 26 07 ba bt high zero_14 15 (2.475) Cm (3:131)
%
m/z
FIG. 4A
FIG. 4B

75g-rna

II_082806_ms2_16 67 (3.429) Cm (19:128)

Pusher Freq 14706

FIG. 5A

II_082806_ms2_20 70 (3.484) Cm (29:129)

FIG. 5B

II_082806_ms2_22 72 (3.520) Cm (27:128)

FIG. 5C

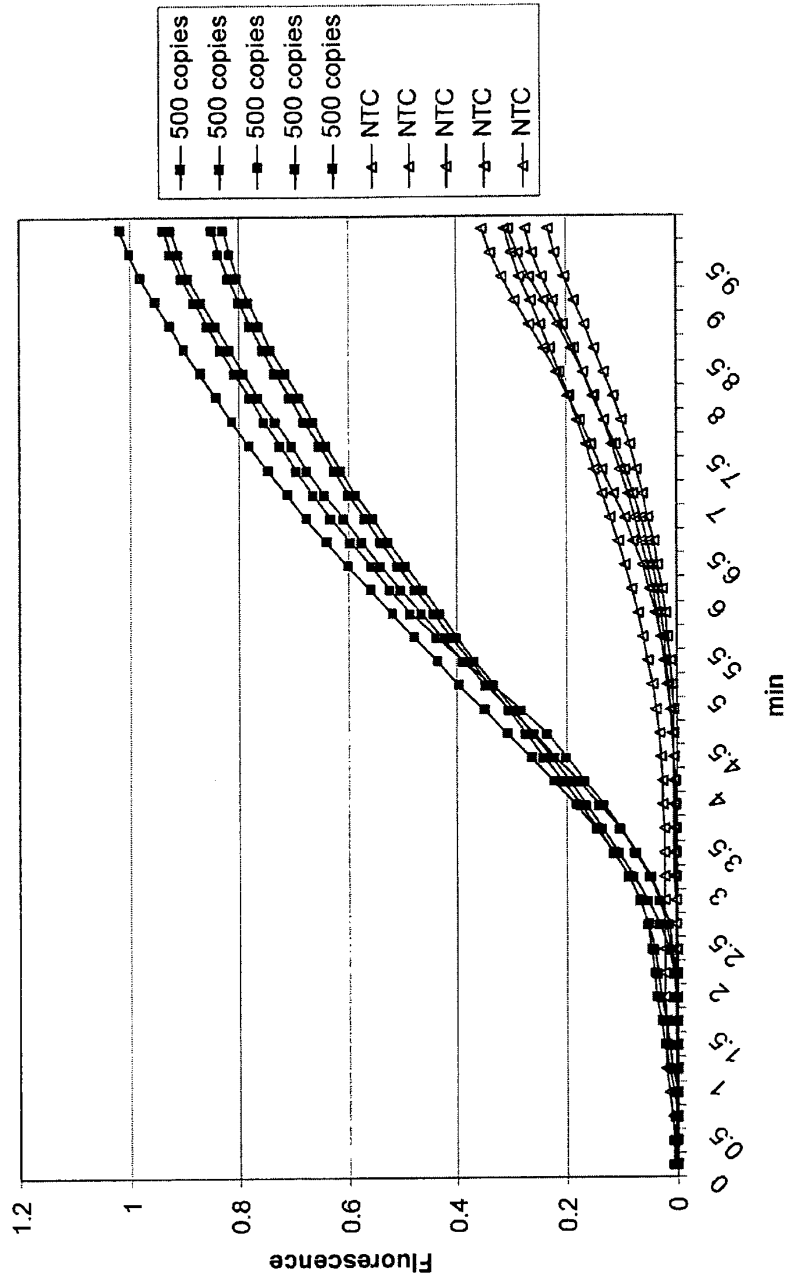


FIG. 6

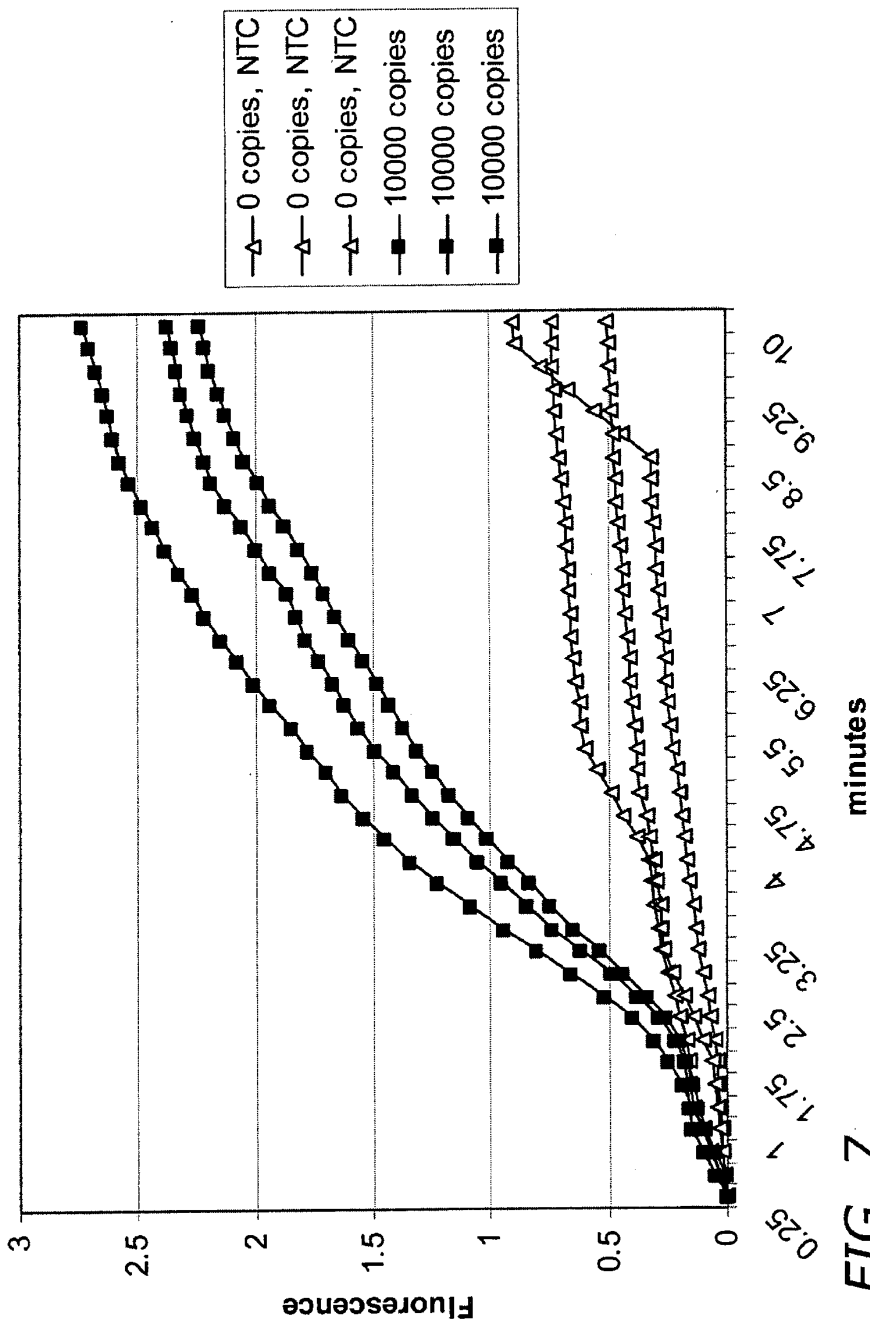


FIG. 7

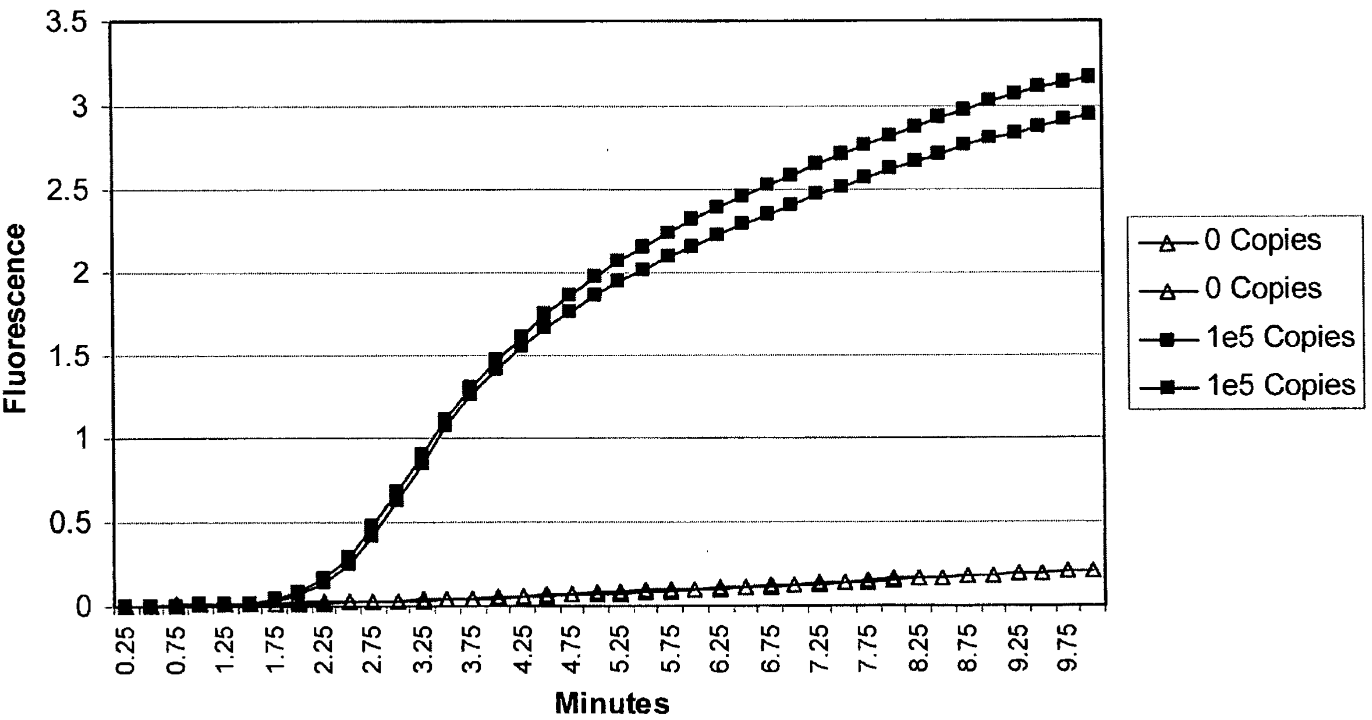


FIG. 8

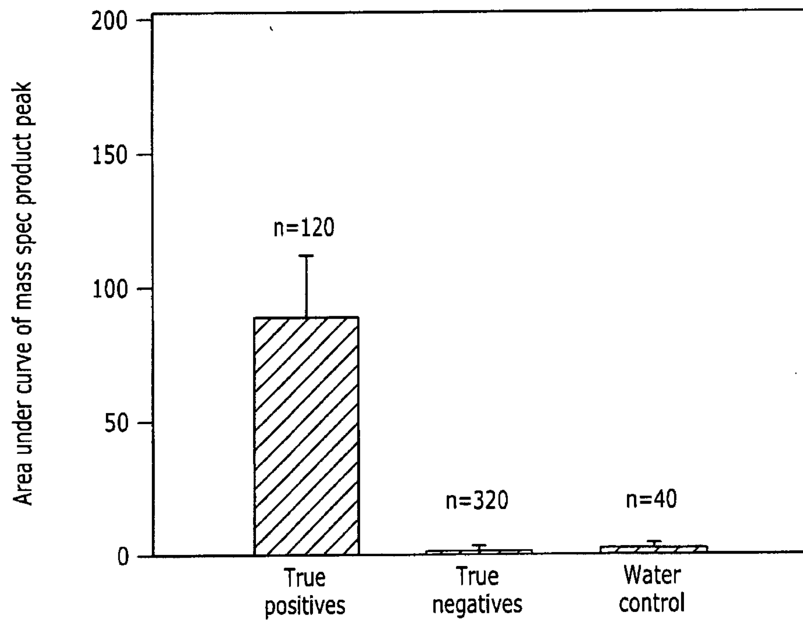
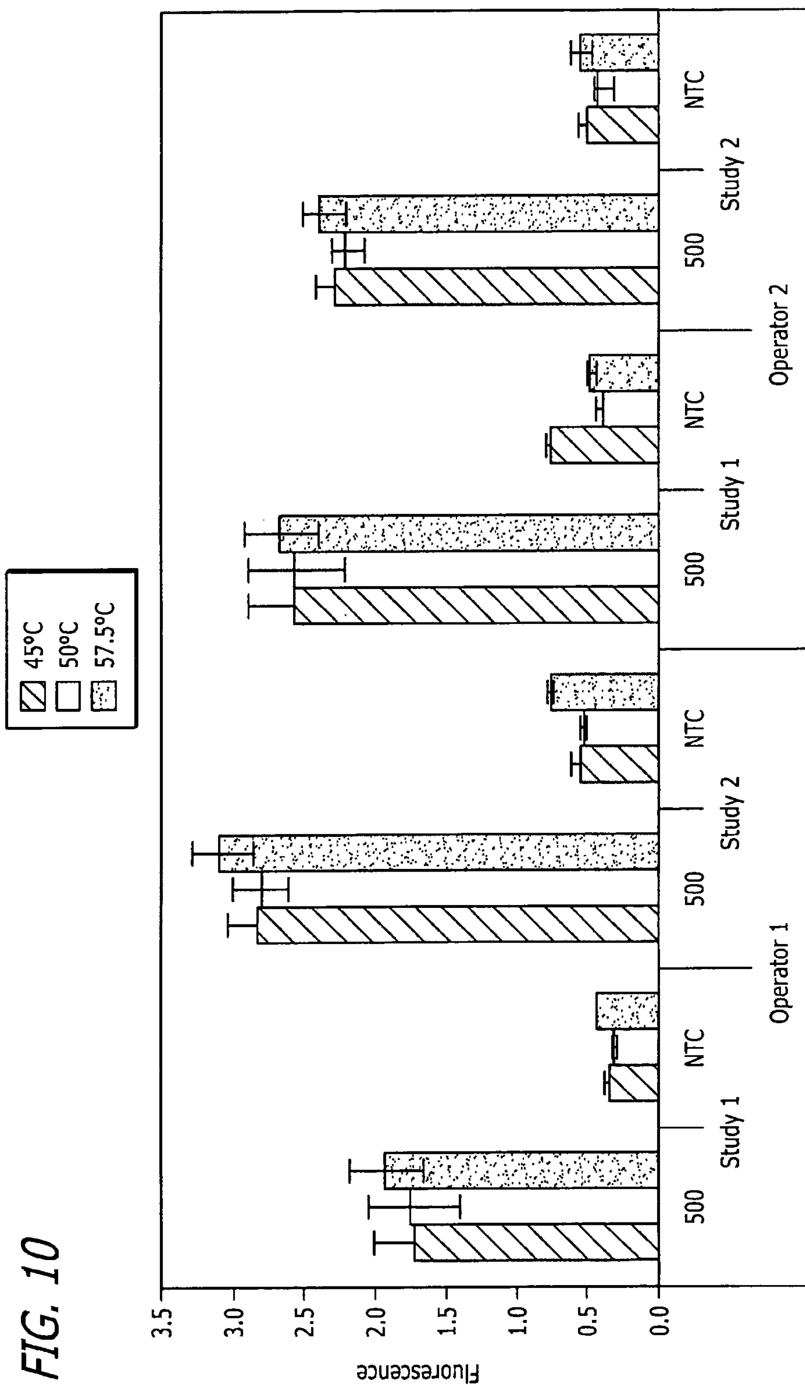


FIG. 9

FIG. 10



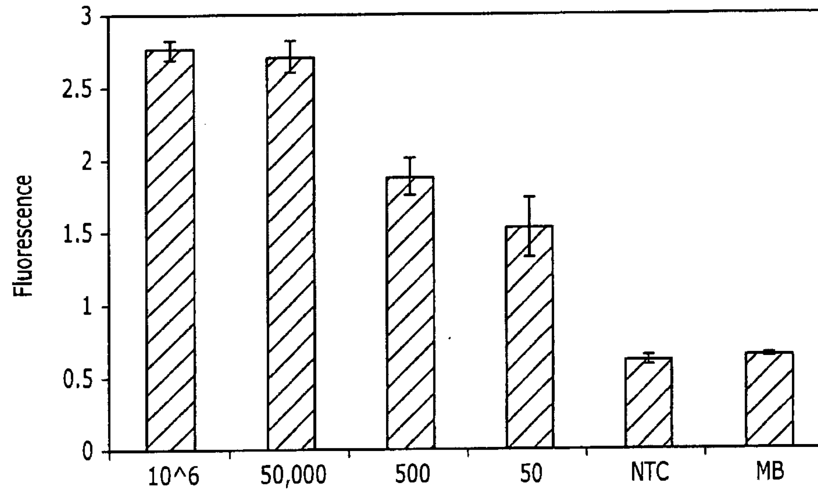


FIG. 11

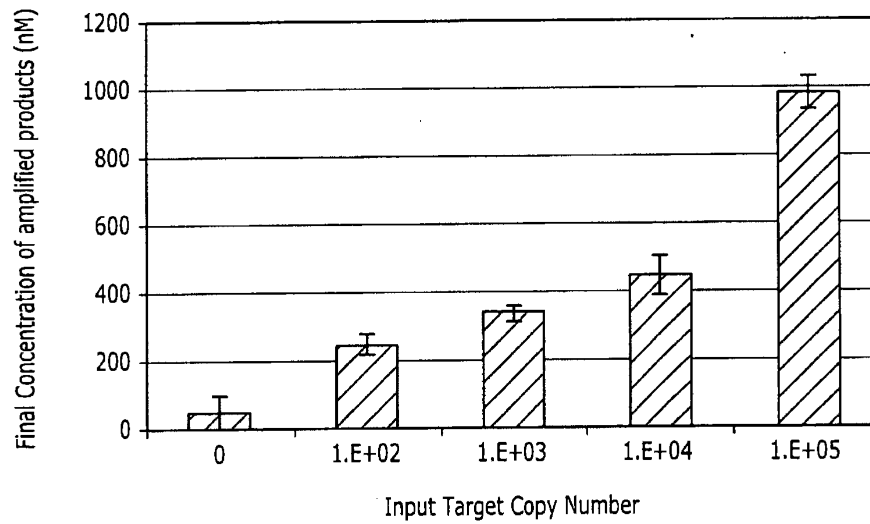


FIG. 12

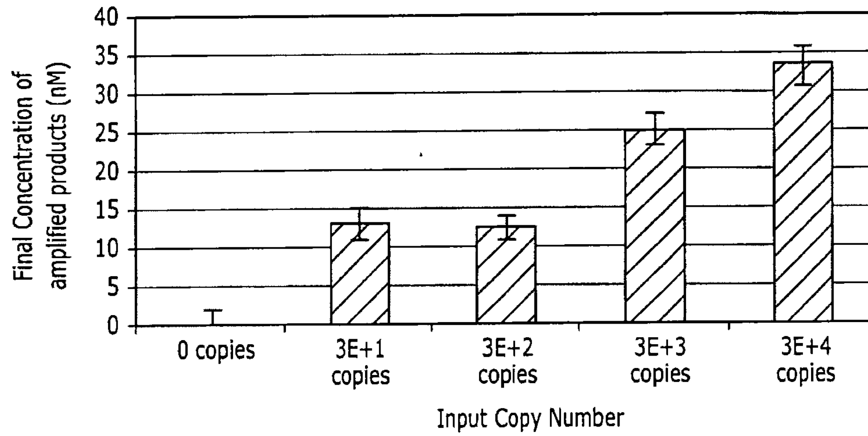


FIG. 13

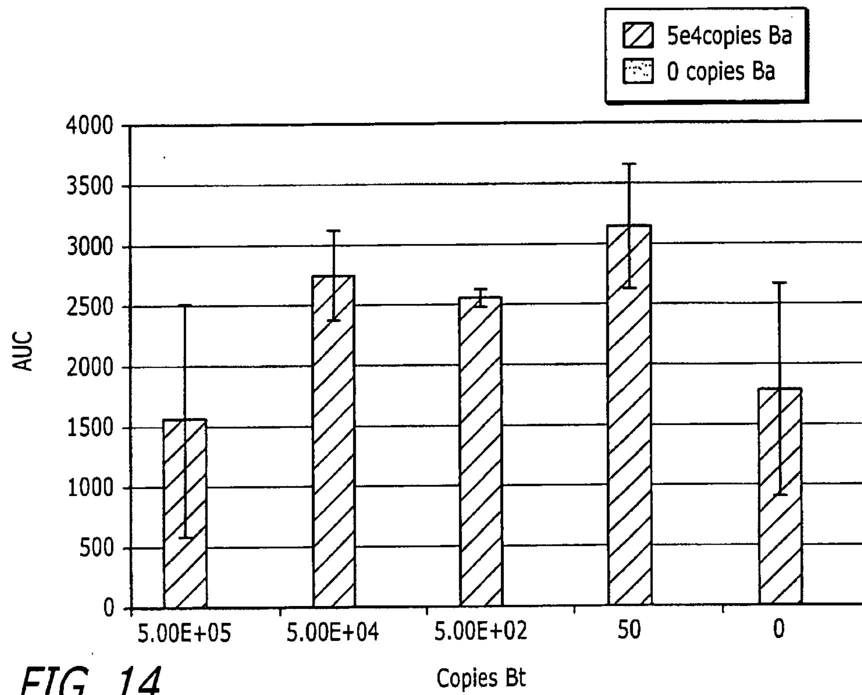


FIG. 14

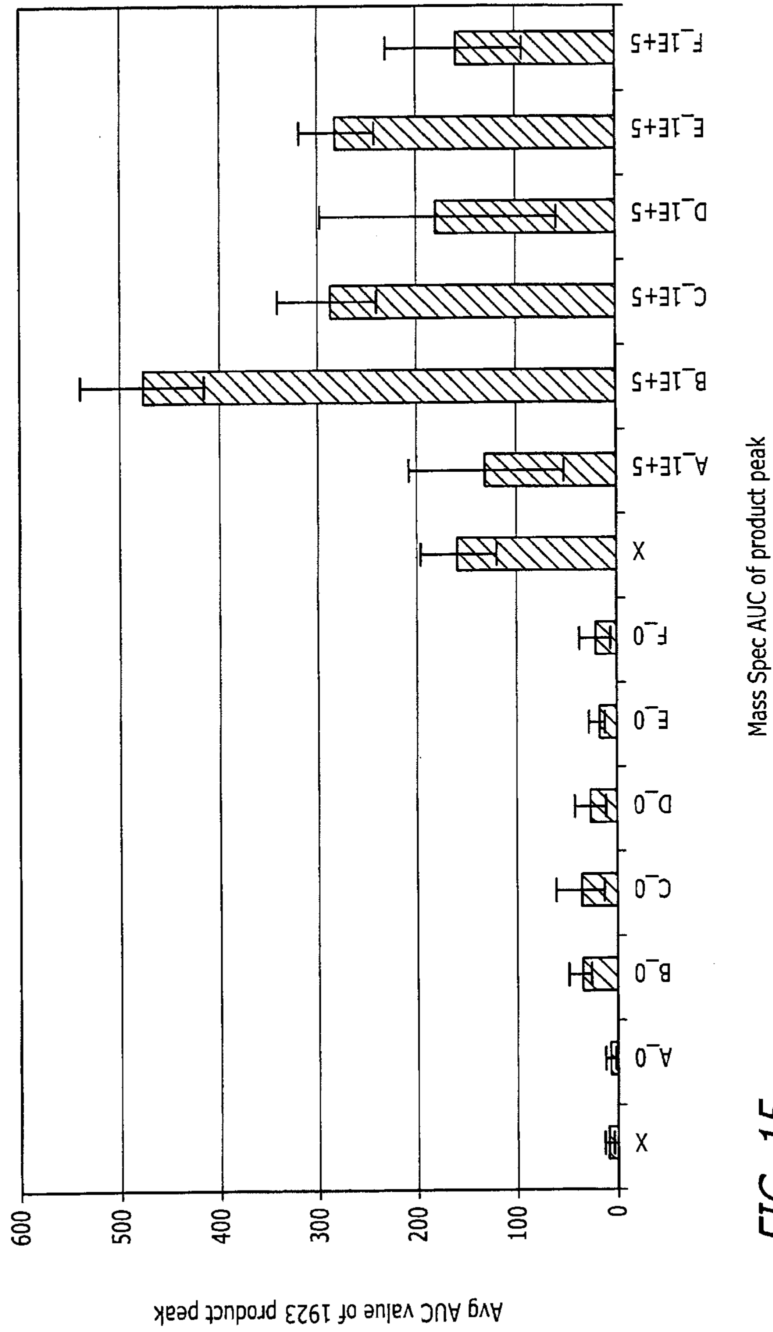


FIG. 15

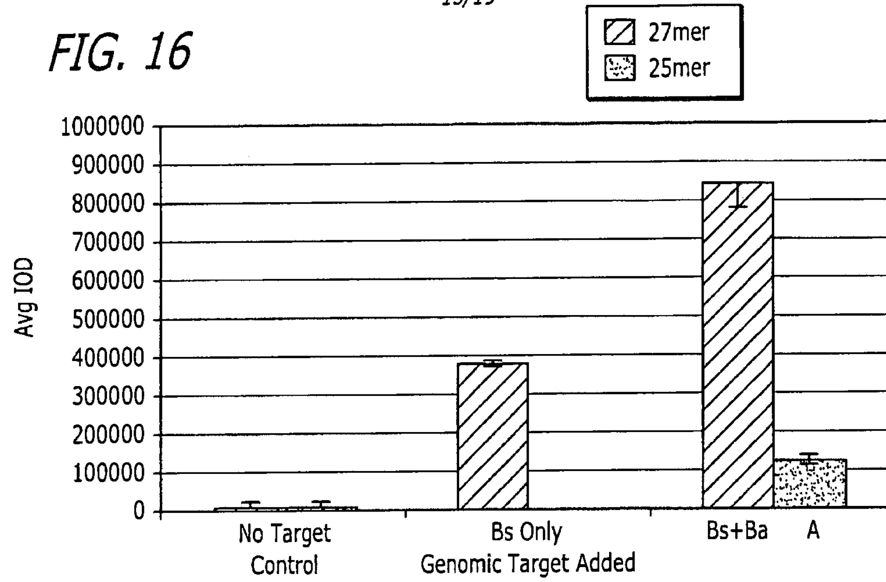


FIG. 16
27mer
25mer
1000000
900000
800000
700000
600000
500000
400000
300000
200000
100000
0
Avg IOD
No Target
Control
Bs Only
Genomic Target Added
Bs+Ba
A

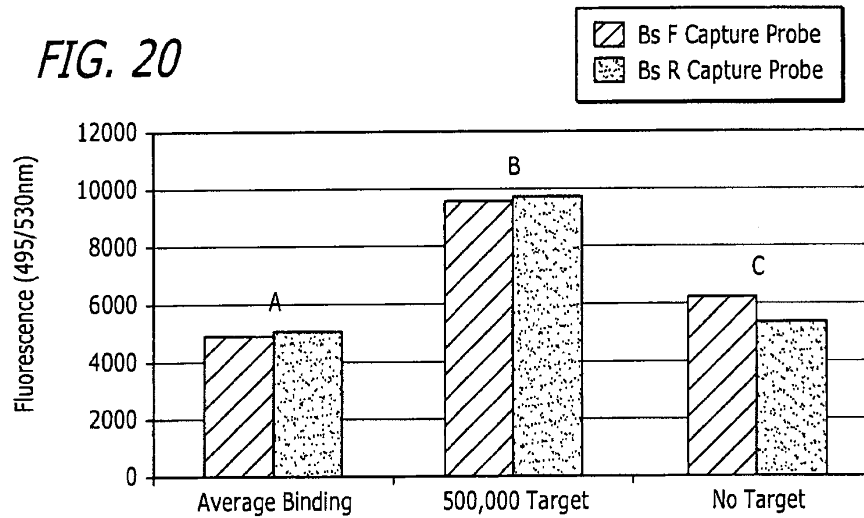


FIG. 20
Bs F Capture Probe
Bs R Capture Probe
12000
10000
8000
6000
4000
2000
0
Fluorescence (495/530nm)
A
B
C
Average Binding
500,000 Target
No Target

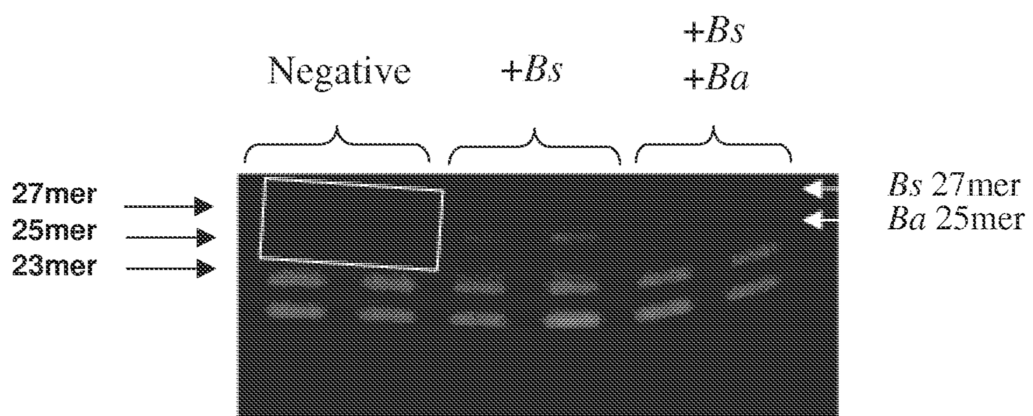


Figure 17

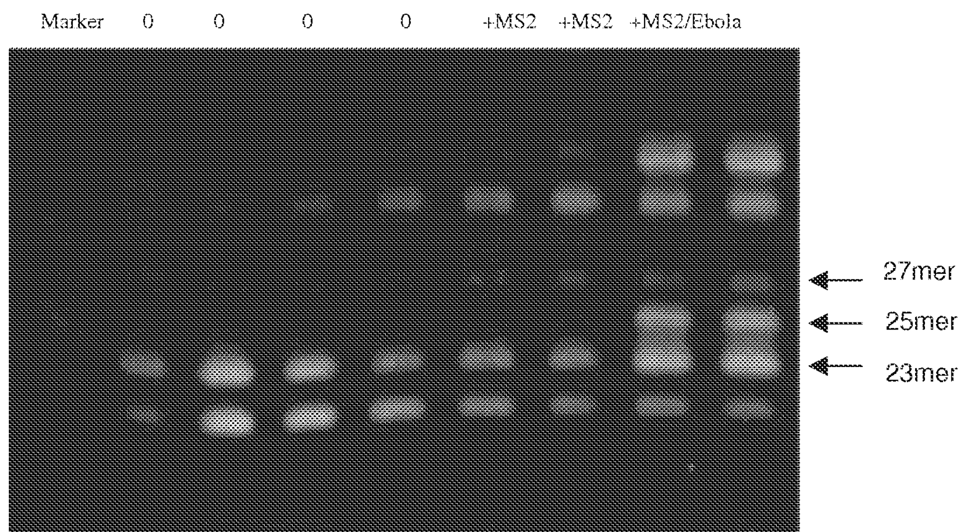


Figure 18

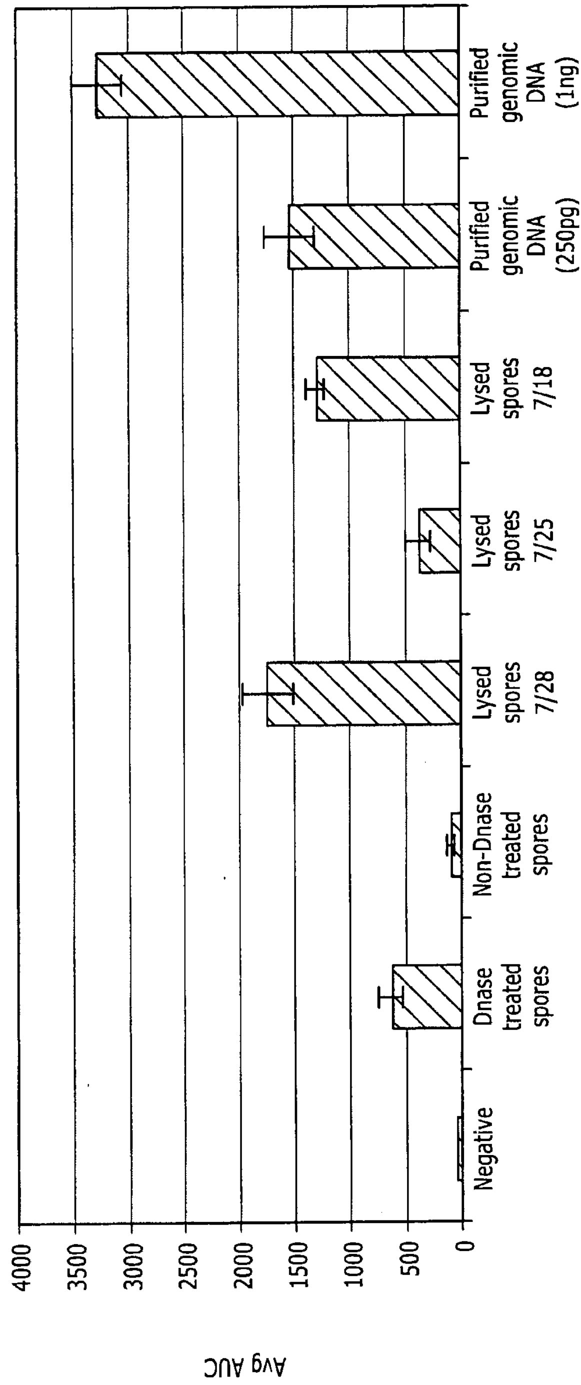


FIG. 19

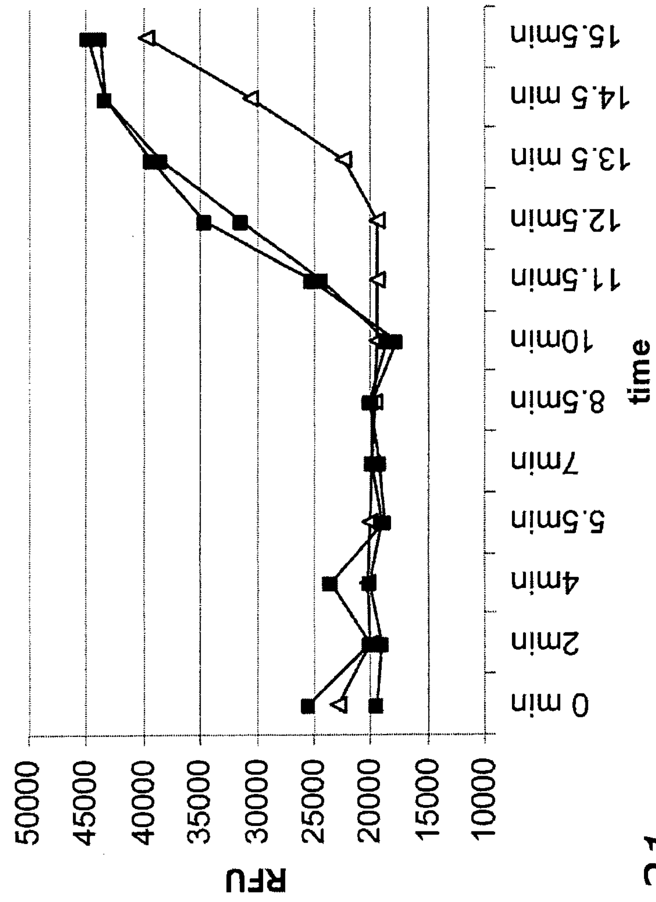


FIG. 21

Electronic Acknowledgement Receipt

EFS ID:	17698237
Application Number:	14067620
International Application Number:	
Confirmation Number:	4288
Title of Invention:	Nicking and Extension Amplification Reaction for the Exponential Amplification of Nucleic Acids
First Named Inventor/Applicant Name:	Brian K. Maples
Customer Number:	26161
Filer:	Ian J.S. Lodovice/Mary Florczak
Filer Authorized By:	Ian J.S. Lodovice
Attorney Docket Number:	30171-0025002 / ITI-002 U
Receipt Date:	18-DEC-2013
Filing Date:	30-OCT-2013
Time Stamp:	15:51:56
Application Type:	Utility under 35 USC 111(a)

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File Listing:

Document Number	Document Description	File Name	File Size(Bytes)/ Message Digest	Multi Part /.zip	Pages (if appl.)
1	Applicant Response to Pre-Exam Formalities Notice	301710025Reply.pdf	53187 e8a2d5a3a94a0f05b34d69a3176aa1696ba876a8	no	1

Warnings:

Information:

2	Specification	301710025002Clean.pdf	279849 89d6c059958fe03cb509d6091e1ff61352755125	no	46
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Information:					
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Information:					
4	Drawings-only black and white line drawings	301710025FIGS.pdf	4206166 51b0d6b0cc5bf07f41069ede605504d192a07948	no	19
Warnings:					
Information:					
		Total Files Size (in bytes):	4820383		

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If a new application is being filed and the application includes the necessary components for a filing date (see 37 CFR 1.53(b)-(d) and MPEP 506), a Filing Receipt (37 CFR 1.54) will be issued in due course and the date shown on this Acknowledgement Receipt will establish the filing date of the application.

National Stage of an International Application under 35 U.S.C. 371
If a timely submission to enter the national stage of an international application is compliant with the conditions of 35 U.S.C. 371 and other applicable requirements a Form PCT/DO/EO/903 indicating acceptance of the application as a national stage submission under 35 U.S.C. 371 will be issued in addition to the Filing Receipt, in due course.

New International Application Filed with the USPTO as a Receiving Office
If a new international application is being filed and the international application includes the necessary components for an international filing date (see PCT Article 11 and MPEP 1810), a Notification of the International Application Number and of the International Filing Date (Form PCT/RO/105) will be issued in due course, subject to prescriptions concerning national security, and the date shown on this Acknowledgement Receipt will establish the international filing date of the application.

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APPLICATION NUMBER	FILING OR 371(C) DATE	FIRST NAMED APPLICANT	ATTY. DOCKET NO./TITLE
14/067,620	10/30/2013	Brian K. Maples	30171-0025002 / ITI-002 U

CONFIRMATION NO. 4288

26161
FISH & RICHARDSON P.C. (BO)
P.O. BOX 1022
MINNEAPOLIS, MN 55440-1022

FORMALITIES LETTER

OC000000065468608

Date Mailed: 12/16/2013

NOTICE TO FILE CORRECTED APPLICATION PAPERS

Filing Date Granted

An application number and filing date have been accorded to this application. The application is informal since it does not comply with the regulations for the reason(s) indicated below. Applicant is given TWO MONTHS from the date of this Notice within which to correct the informalities indicated below. Extensions of time may be obtained by filing a petition accompanied by the extension fee under the provisions of 37 CFR 1.136(a).

The required item(s) identified below must be timely submitted to avoid abandonment:

- A substitute specification in compliance with 37 CFR 1.52, 1.121(b)(3), and 1.125, is required. The substitute specification must be submitted with markings and be accompanied by a clean version (without markings) as set forth in 37 CFR 1.125(c) and a statement that the substitute specification contains no new matter (see 37 CFR 1.125(b)). The specification, claims, and/or abstract page(s) submitted is not acceptable and cannot be scanned or properly stored because:
  - The application contains drawings, but the specification does not contain a brief description of the several views of the drawings as required by 37 CFR 1.74 and 37 CFR 1.77(b)(7).
- Replacement drawings in compliance with 37 CFR 1.84 and 37 CFR 1.121(d) are required. The drawings submitted are not acceptable because:
  - More than one figure is present and each figure is not labeled "Fig." with a consecutive Arabic numeral (1, 2, etc.) or an Arabic numeral and capital letter in the English alphabet (A, B, etc.)(see 37 CFR 1.84(u)(1)). See Figure(s) 5. A brief description of the several views of the drawings (see 37 CFR 1.74) should be added or amended to correspond to the corrected numbering of the figures. See also 37 CFR 1.77(b)(7).

Applicant is cautioned that correction of the above items may cause the specification and drawings page count to exceed 100 pages. If the specification and drawings exceed 100 pages, applicant will need to submit the required application size fee.

Replies must be received in the USPTO within the set time period or must include a proper Certificate of Mailing or Transmission under 37 CFR 1.8 with a mailing or transmission date within the set time period. For more information and a suggested format, see Form PTO/SB/92 and MPEP 512.

Replies should be mailed to:

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APPLICATION NUMBER	FILING or 371(c) DATE	GRP ART UNIT	FIL FEE REC'D	ATTY.DOCKET.NO	TOT CLAIMS	IND CLAIMS
14/067,620	10/30/2013	1637	2620	30171-0025002 / ITI-002 U	29	3

CONFIRMATION NO. 4288

26161
FISH & RICHARDSON P.C. (BO)
P.O. BOX 1022
MINNEAPOLIS, MN 55440-1022

FILING RECEIPT

OC000000065468607

Date Mailed: 12/16/2013

Receipt is acknowledged of this non-provisional patent application. The application will be taken up for examination in due course. Applicant will be notified as to the results of the examination. Any correspondence concerning the application must include the following identification information: the U.S. APPLICATION NUMBER, FILING DATE, NAME OF APPLICANT, and TITLE OF INVENTION. Fees transmitted by check or draft are subject to collection. Please verify the accuracy of the data presented on this receipt. **If an error is noted on this Filing Receipt, please submit a written request for a Filing Receipt Correction. Please provide a copy of this Filing Receipt with the changes noted thereon. If you received a "Notice to File Missing Parts" for this application, please submit any corrections to this Filing Receipt with your reply to the Notice. When the USPTO processes the reply to the Notice, the USPTO will generate another Filing Receipt incorporating the requested corrections**

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Andrew P. Miller, San Diego, CA;
Jarrod Provins, Dana Point, CA;
Richard Roth, Carlsbad, CA;
Jeffrey Mandell, San Diego, CA;

Applicant(s)

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Assignment For Published Patent Application

Ionian Technologies, Inc., San Diego, CA

Power of Attorney: The patent practitioners associated with Customer Number 26161

Domestic Priority data as claimed by applicant

This application is a CON of 11/778,018 07/14/2007

Foreign Applications for which priority is claimed (You may be eligible to benefit from the **Patent Prosecution Highway** program at the USPTO. Please see http://www.uspto.gov for more information.) - None.
Foreign application information must be provided in an Application Data Sheet in order to constitute a claim to foreign priority. See 37 CFR 1.55 and 1.76.

If Required, Foreign Filing License Granted: 11/21/2013
The country code and number of your priority application, to be used for filing abroad under the Paris Convention, is **US 14/067,620**
Projected Publication Date: To Be Determined - pending completion of Corrected Papers

Non-Publication Request: No

Early Publication Request: No
Title

Nicking and Extension Amplification Reaction for the Exponential Amplification of Nucleic Acids

Preliminary Class

435

Statement under 37 CFR 1.55 or 1.78 for AIA (First Inventor to File) Transition Applications: No

PROTECTING YOUR INVENTION OUTSIDE THE UNITED STATES

Since the rights granted by a U.S. patent extend only throughout the territory of the United States and have no effect in a foreign country, an inventor who wishes patent protection in another country must apply for a patent in a specific country or in regional patent offices. Applicants may wish to consider the filing of an international application under the Patent Cooperation Treaty (PCT). An international (PCT) application generally has the same effect as a regular national patent application in each PCT-member country. The PCT process **simplifies** the filing of patent applications on the same invention in member countries, but **does not result** in a grant of "an international patent" and does not eliminate the need of applicants to file additional documents and fees in countries where patent protection is desired.

Almost every country has its own patent law, and a person desiring a patent in a particular country must make an application for patent in that country in accordance with its particular laws. Since the laws of many countries differ in various respects from the patent law of the United States, applicants are advised to seek guidance from specific foreign countries to ensure that patent rights are not lost prematurely.

Applicants also are advised that in the case of inventions made in the United States, the Director of the USPTO must issue a license before applicants can apply for a patent in a foreign country. The filing of a U.S. patent application serves as a request for a foreign filing license. The application's filing receipt contains further information and guidance as to the status of applicant's license for foreign filing.

Applicants may wish to consult the USPTO booklet, "General Information Concerning Patents" (specifically, the section entitled "Treaties and Foreign Patents") for more information on timeframes and deadlines for filing foreign patent applications. The guide is available either by contacting the USPTO Contact Center at 800-786-9199, or it can be viewed on the USPTO website at http://www.uspto.gov/web/offices/pac/doc/general/index.html.

For information on preventing theft of your intellectual property (patents, trademarks and copyrights), you may wish to consult the U.S. Government website, http://www.stopfakes.gov. Part of a Department of Commerce initiative, this website includes self-help "toolkits" giving innovators guidance on how to protect intellectual property in specific countries such as China, Korea and Mexico. For questions regarding patent enforcement issues, applicants may call the U.S. Government hotline at 1-866-999-HALT (1-866-999-4258).

LICENSE FOR FOREIGN FILING UNDER

Title 35, United States Code, Section 184

Title 37, Code of Federal Regulations, 5.11 & 5.15

GRANTED

The applicant has been granted a license under 35 U.S.C. 184, if the phrase "IF REQUIRED, FOREIGN FILING LICENSE GRANTED" followed by a date appears on this form. Such licenses are issued in all applications where the conditions for issuance of a license have been met, regardless of whether or not a license may be required as set forth in 37 CFR 5.15. The scope and limitations of this license are set forth in 37 CFR 5.15(a) unless an earlier license has been issued under 37 CFR 5.15(b). The license is subject to revocation upon written notification. The date indicated is the effective date of the license, unless an earlier license of similar scope has been granted under 37 CFR 5.13 or 5.14.

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The grant of a license does not in any way lessen the responsibility of a licensee for the security of the subject matter as imposed by any Government contract or the provisions of existing laws relating to espionage and the national security or the export of technical data. Licensees should apprise themselves of current regulations especially with respect to certain countries, of other agencies, particularly the Office of Defense Trade Controls, Department of State (with respect to Arms, Munitions and Implements of War (22 CFR 121-128)); the Bureau of Industry and Security, Department of Commerce (15 CFR parts 730-774); the Office of Foreign AssetsControl, Department of Treasury (31 CFR Parts 500+) and the Department of Energy.

NOT GRANTED

No license under 35 U.S.C. 184 has been granted at this time, if the phrase "IF REQUIRED, FOREIGN FILING LICENSE GRANTED" DOES NOT appear on this form. Applicant may still petition for a license under 37 CFR 5.12, if a license is desired before the expiration of 6 months from the filing date of the application. If 6 months has lapsed from the filing date of this application and the licensee has not received any indication of a secrecy order under 35 U.S.C. 181, the licensee may foreign file the application pursuant to 37 CFR 5.15(b).

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UNITED STATES DEPARTMENT OF COMMERCE
United States Patent and Trademark Office
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www.uspto.gov

APPLICATION NUMBER	FILING OR 371(C) DATE	FIRST NAMED APPLICANT	ATTY. DOCKET NO./TITLE
14/067,620	10/30/2013	Brian K. Maples	30171-0025002 / ITI-002 U

CONFIRMATION NO. 4288

26161
FISH & RICHARDSON P.C. (BO)
P.O. BOX 1022
MINNEAPOLIS, MN 55440-1022

WITHDRAWAL NOTICE

Date Mailed: 12/16/2013

Letter Regarding a New Notice and/or the Status of the Application

If a new notice or Filing Receipt is enclosed, applicant may disregard the previous notice mailed on 12/02/2013. The time period for reply runs from the mail date of the new notice. Within the time period for reply, applicant is required to file a reply in compliance with the requirements set forth in the new notice to avoid abandonment of the application.

Registered users of EFS-Web may alternatively submit their reply to this notice via EFS-Web. https://sportal.uspto.gov/authenticate/AuthenticateUserLocalEPF.html

For more information about EFS-Web please call the USPTO Electronic Business Center at **1-866-217-9197** or visit our website at http://www.uspto.gov/ebc.

If the reply is not filed electronically via EFS-Web, the reply must be accompanied by a copy of the new notice.

If the Office previously granted a petition to withdraw the holding of abandonment or a petition to revive under 37 CFR 1.137, the status of the application has been returned to pending status.

/nton/

Office of Data Management, Application Assistance Unit (571) 272-4000, or (571) 272-4200, or 1-888-786-0101

PATENT APPLICATION FEE DETERMINATION RECORD

Substitute for Form PTO-875

Application or Docket Number
14/067,620

APPLICATION AS FILED - PART I

FOR	NUMBER FILED (Column 1)	NUMBER EXTRA (Column 2)	SMALL ENTITY RATE($)	SMALL ENTITY FEE($)	OR	OTHER THAN SMALL ENTITY RATE($)	OTHER THAN SMALL ENTITY FEE($)
BASIC FEE (37 CFR 1.16(a), (b), or (c))	N/A	N/A	N/A			N/A	280
SEARCH FEE (37 CFR 1.16(k), (i), or (m))	N/A	N/A	N/A			N/A	600
EXAMINATION FEE (37 CFR 1.16(o), (p), or (q))	N/A	N/A	N/A			N/A	720
TOTAL CLAIMS (37 CFR 1.16(i))	29 minus 20 =	* 9			OR	x 80 =	720
INDEPENDENT CLAIMS (37 CFR 1.16(h))	3 minus 3 =	*				x 420 =	0.00
APPLICATION SIZE FEE (37 CFR 1.16(s))	If the specification and drawings exceed 100 sheets of paper, the application size fee due is $310 ($155 for small entity) for each additional 50 sheets or fraction thereof. See 35 U.S.C. 41(a)(1)(G) and 37 CFR 1.16(s).						0.00
MULTIPLE DEPENDENT CLAIM PRESENT (37 CFR 1.16(j))							0.00
			TOTAL			TOTAL	2320

* If the difference in column 1 is less than zero, enter "0" in column 2.

APPLICATION AS AMENDED - PART II

AMENDMENT A

	CLAIMS REMAINING AFTER AMENDMENT (Column 1)		HIGHEST NUMBER PREVIOUSLY PAID FOR (Column 2)	PRESENT EXTRA (Column 3)	SMALL ENTITY RATE($)	SMALL ENTITY ADDITIONAL FEE($)	OR	OTHER THAN SMALL ENTITY RATE($)	OTHER THAN SMALL ENTITY ADDITIONAL FEE($)
Total (37 CFR 1.16(i))	*	Minus	**	=	x =		OR	x =	
Independent (37 CFR 1.16(h))	*	Minus	***	=	x =		OR	x =	
Application Size Fee (37 CFR 1.16(s))									
FIRST PRESENTATION OF MULTIPLE DEPENDENT CLAIM (37 CFR 1.16(j))							OR		
					TOTAL ADD'L FEE		OR	TOTAL ADD'L FEE	

AMENDMENT B

	CLAIMS REMAINING AFTER AMENDMENT (Column 1)		HIGHEST NUMBER PREVIOUSLY PAID FOR (Column 2)	PRESENT EXTRA (Column 3)	SMALL ENTITY RATE($)	SMALL ENTITY ADDITIONAL FEE($)	OR	OTHER THAN SMALL ENTITY RATE($)	OTHER THAN SMALL ENTITY ADDITIONAL FEE($)
Total (37 CFR 1.16(i))	*	Minus	**	=	x =		OR	x =	
Independent (37 CFR 1.16(h))	*	Minus	***	=	x =		OR	x =	
Application Size Fee (37 CFR 1.16(s))									
FIRST PRESENTATION OF MULTIPLE DEPENDENT CLAIM (37 CFR 1.16(j))							OR		
					TOTAL ADD'L FEE		OR	TOTAL ADD'L FEE	

* If the entry in column 1 is less than the entry in column 2, write "0" in column 3.
** If the "Highest Number Previously Paid For" IN THIS SPACE is less than 20, enter "20".
*** If the "Highest Number Previously Paid For" IN THIS SPACE is less than 3, enter "3".
The "Highest Number Previously Paid For" (Total or Independent) is the highest found in the appropriate box in column 1.

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

Applicant	:	Ionian Technologies Inc.	Art Unit	:	1637
Serial No.	:	14/067,620	Examiner	:	Unknown
Filed	:	October 30, 2013	Conf. No.	:	4288
Title	:	NICKING AND EXTENSION AMPLIFICATION REACTION FOR THE EXPONENTIAL AMPLIFICATION OF NUCLEIC ACIDS			

Commissioner for Patents
P.O. Box 1450
Alexandria, VA 22313-1450

RESPONSE TO NOTICE TO FILE CORRECTED APPLICATION PAPERS DATED DECEMBER 2, 2013

The applicant as a large entity submits:

☒ Replacement drawings (19 sheets) in compliance with 37 CFR §1.84.

The applicant understands that this perfects the application and no additional papers or filing fees are required.

Please apply any necessary charges or credits to Deposit Account 06-1050, referencing the above attorney docket number.

Respectfully submitted,

Date: December 6, 2013

/Ian J.S. Lodovice, Reg. No. 59,749/
Ian J. Lodovice
Reg. No. 59,749

Customer Number 26161
Fish & Richardson P.C.
Telephone: (617) 542-5070
Facsimile: (877) 769-7945

23130369.doc

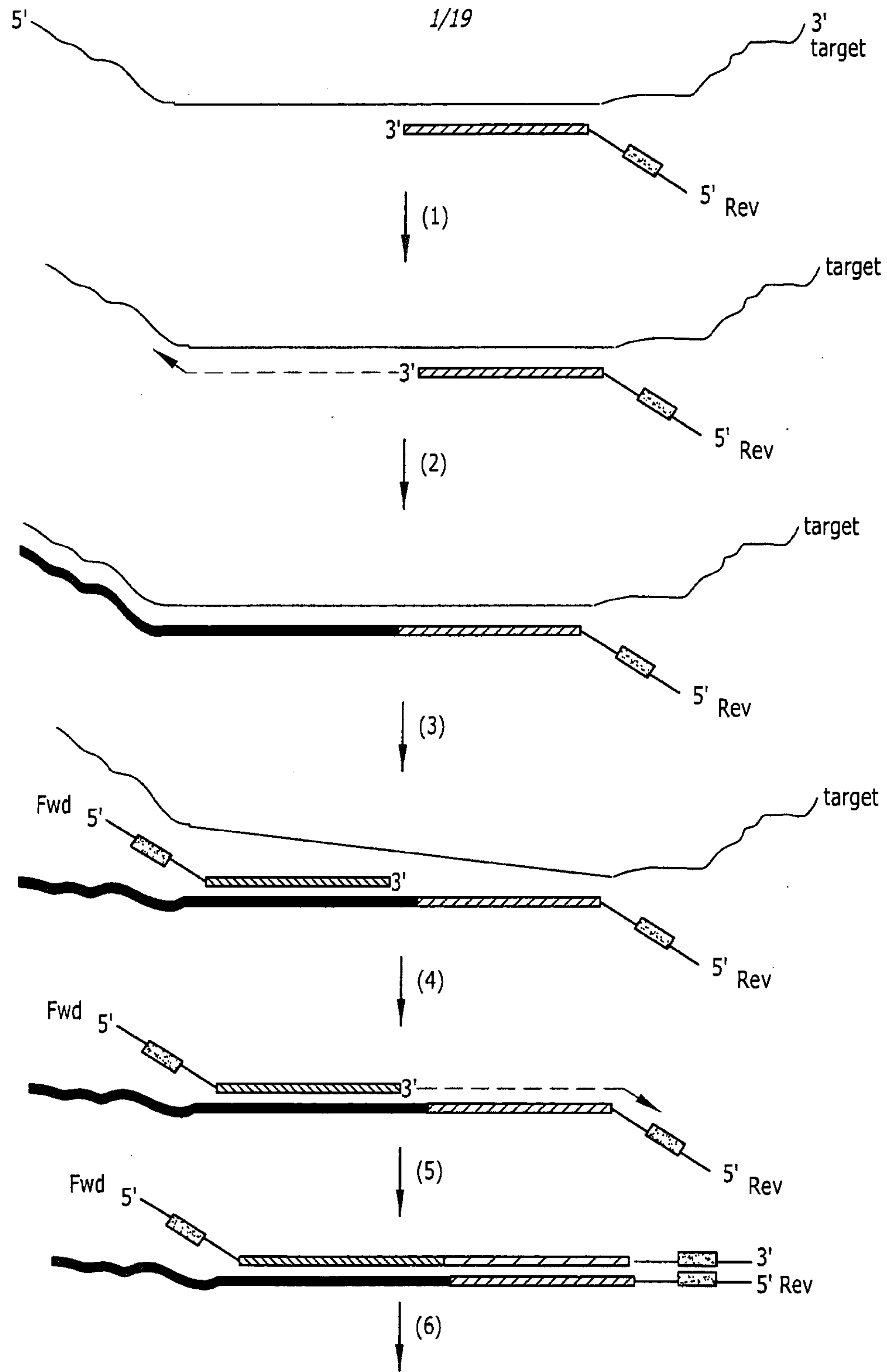


FIG. 1A

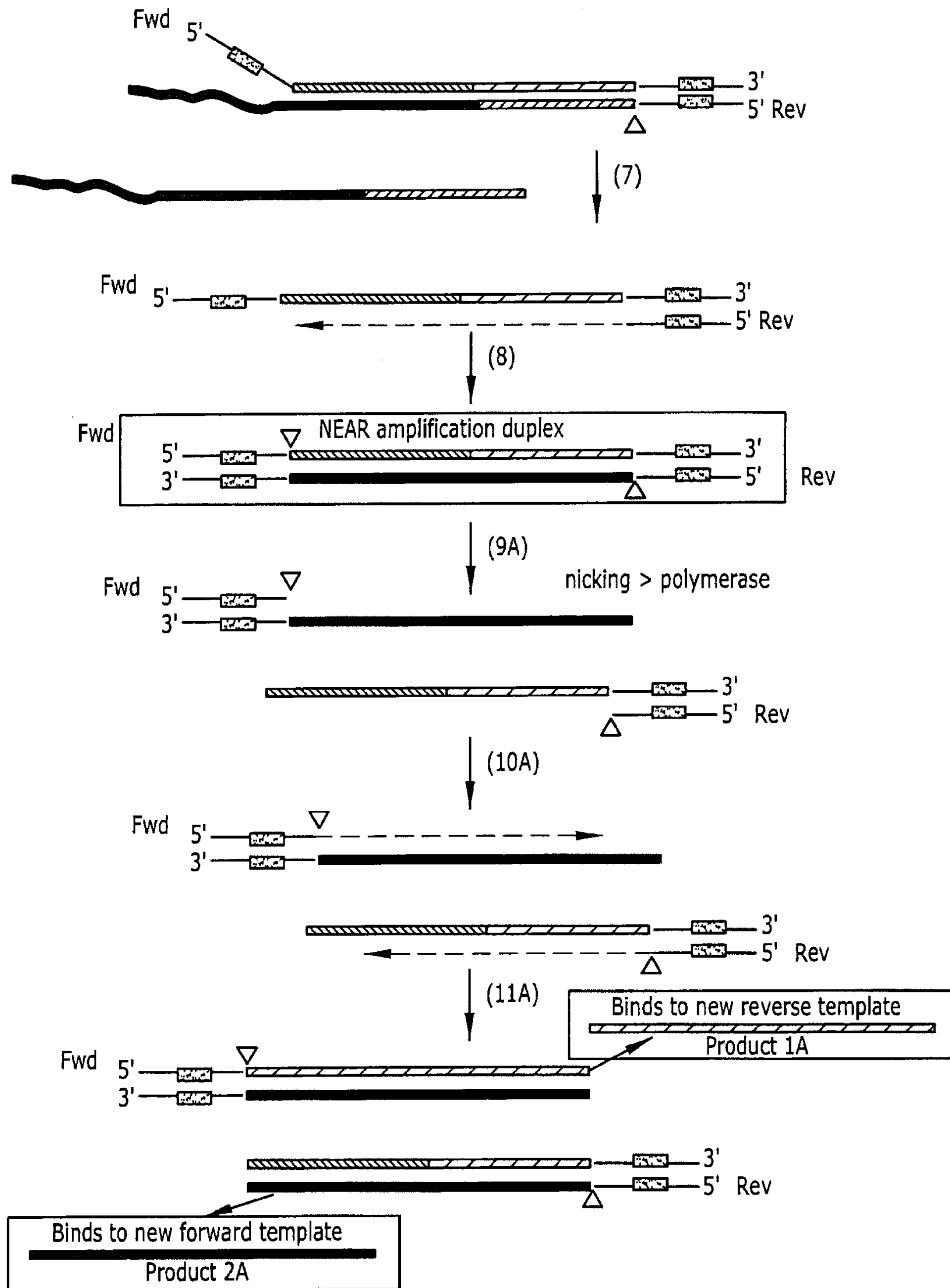


FIG. 1B

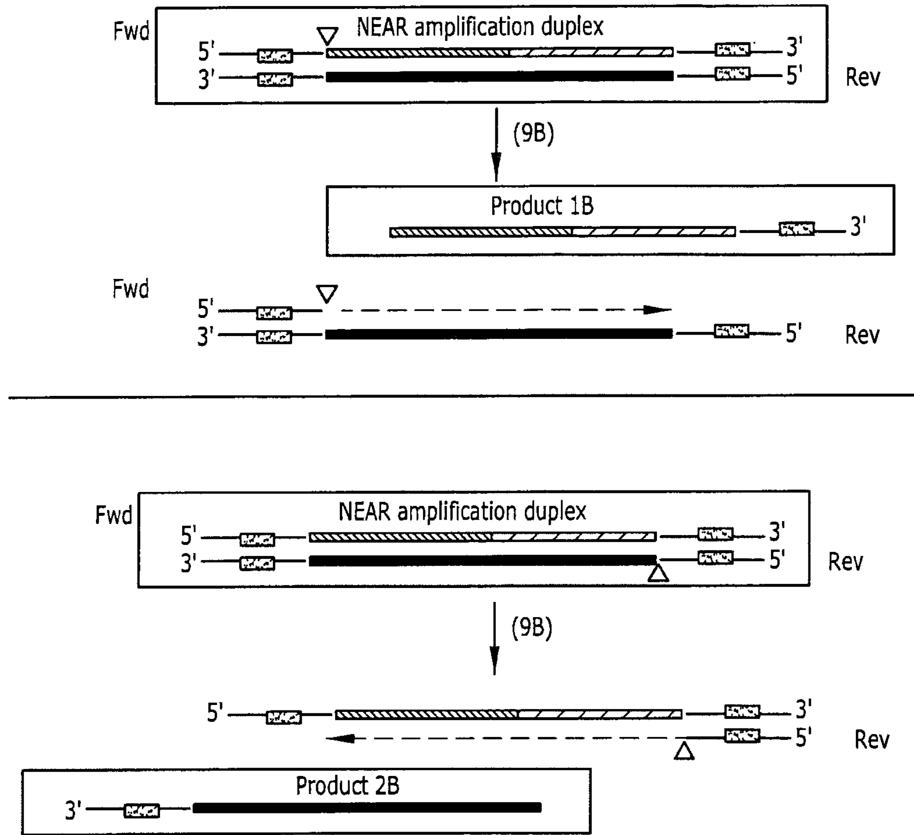


FIG. 1C

FIG. 1D

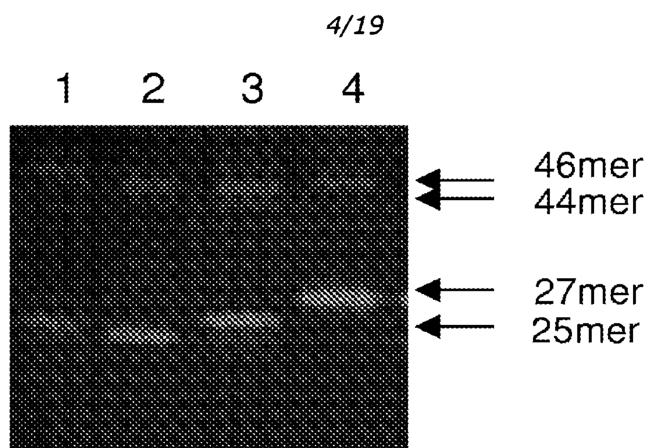


FIG. 2

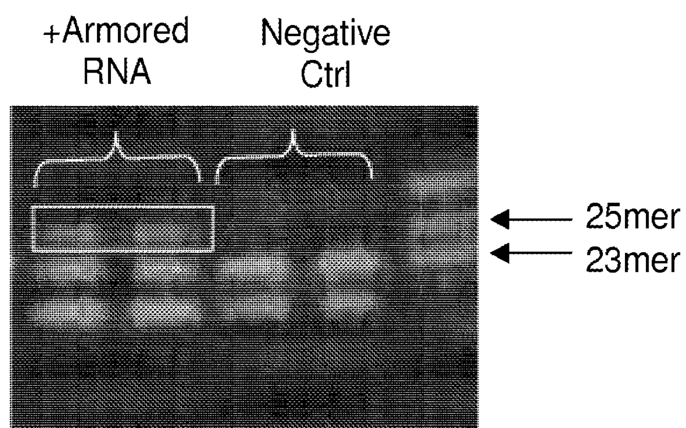


FIG. 3

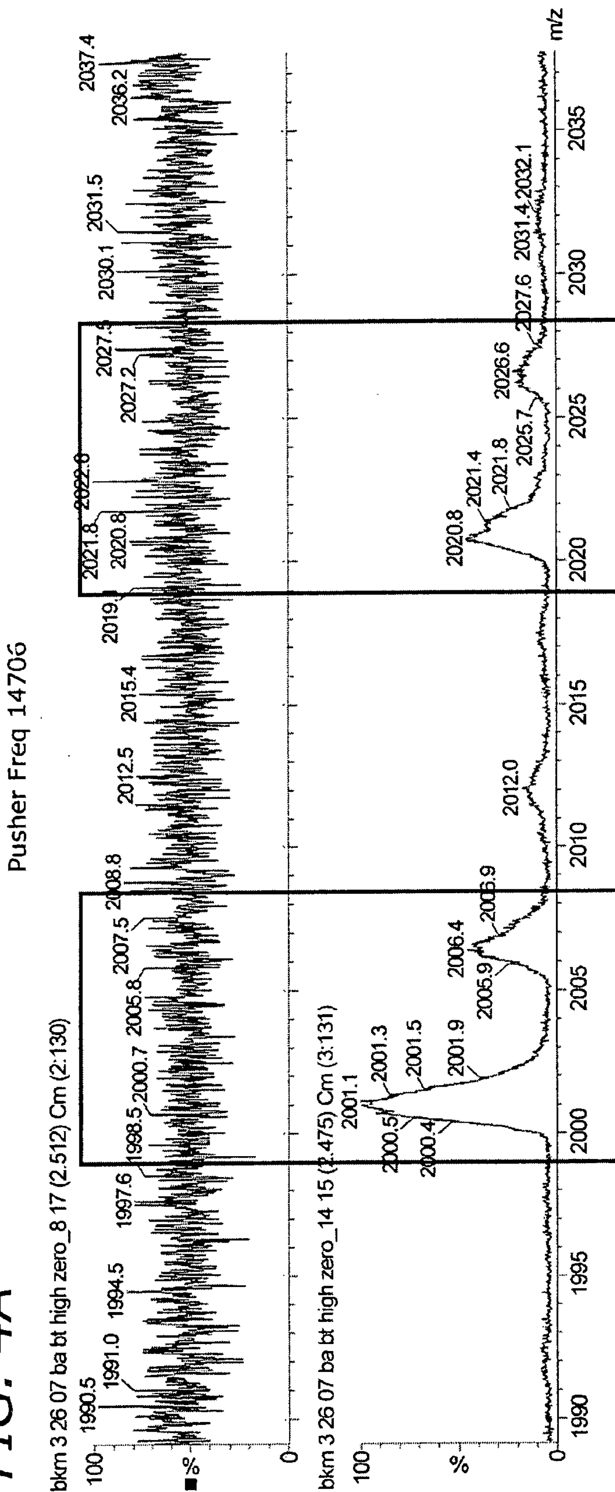
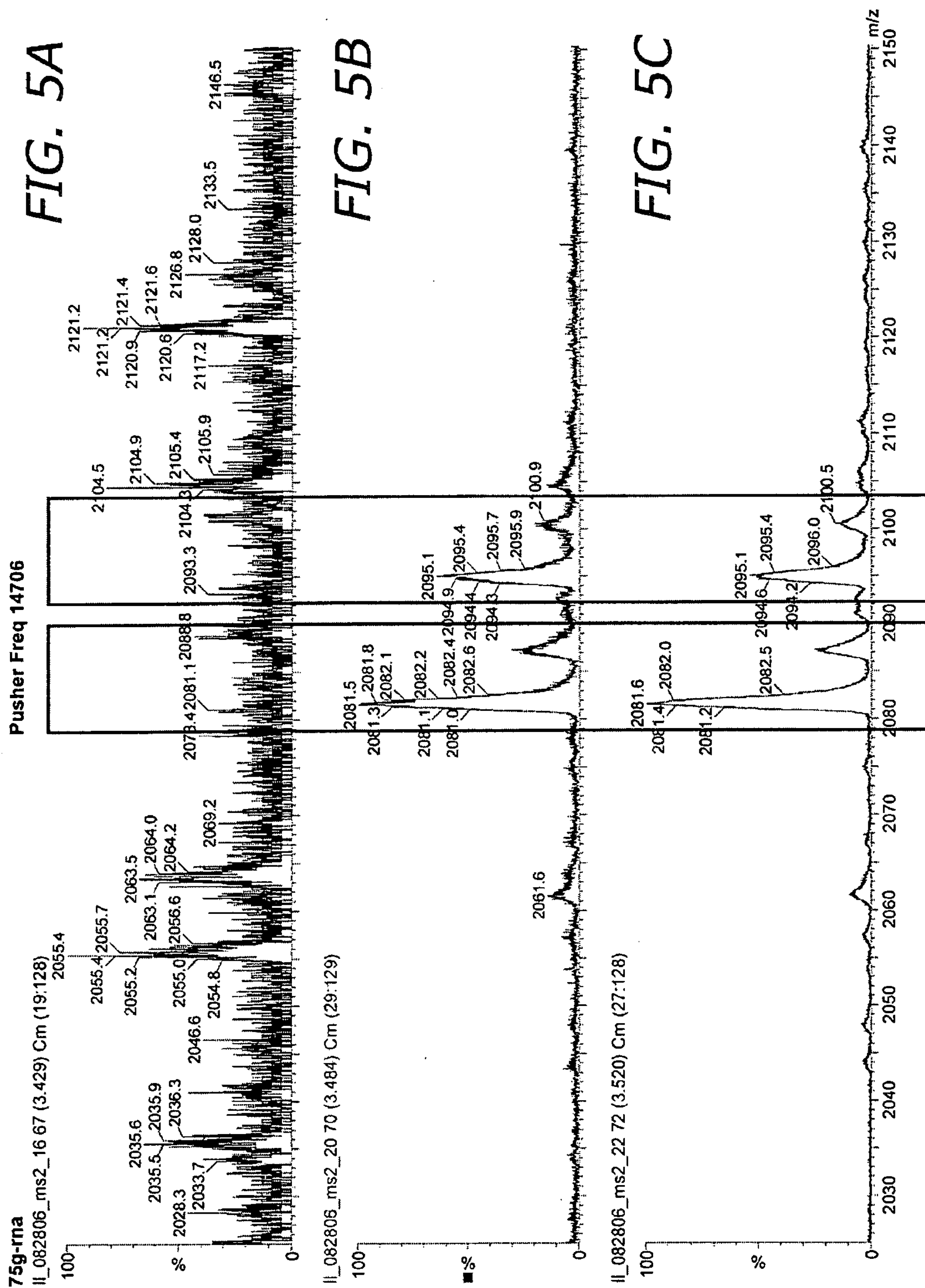


FIG. 4A

FIG. 4B


75g-rna
Il_082806_ms2_16 67 (3.429) Cm (19:128)
Il_082806_ms2_20 70 (3.484) Cm (29:129)
Il_082806_ms2_22 72 (3.520) Cm (27:128)
Pusher Freq 14706
FIG. 5A
FIG. 5B
FIG. 5C
%
100
0
m/z
2030
2040
2050
2060
2070
2080
2090
2100
2110
2120
2130
2140
2150

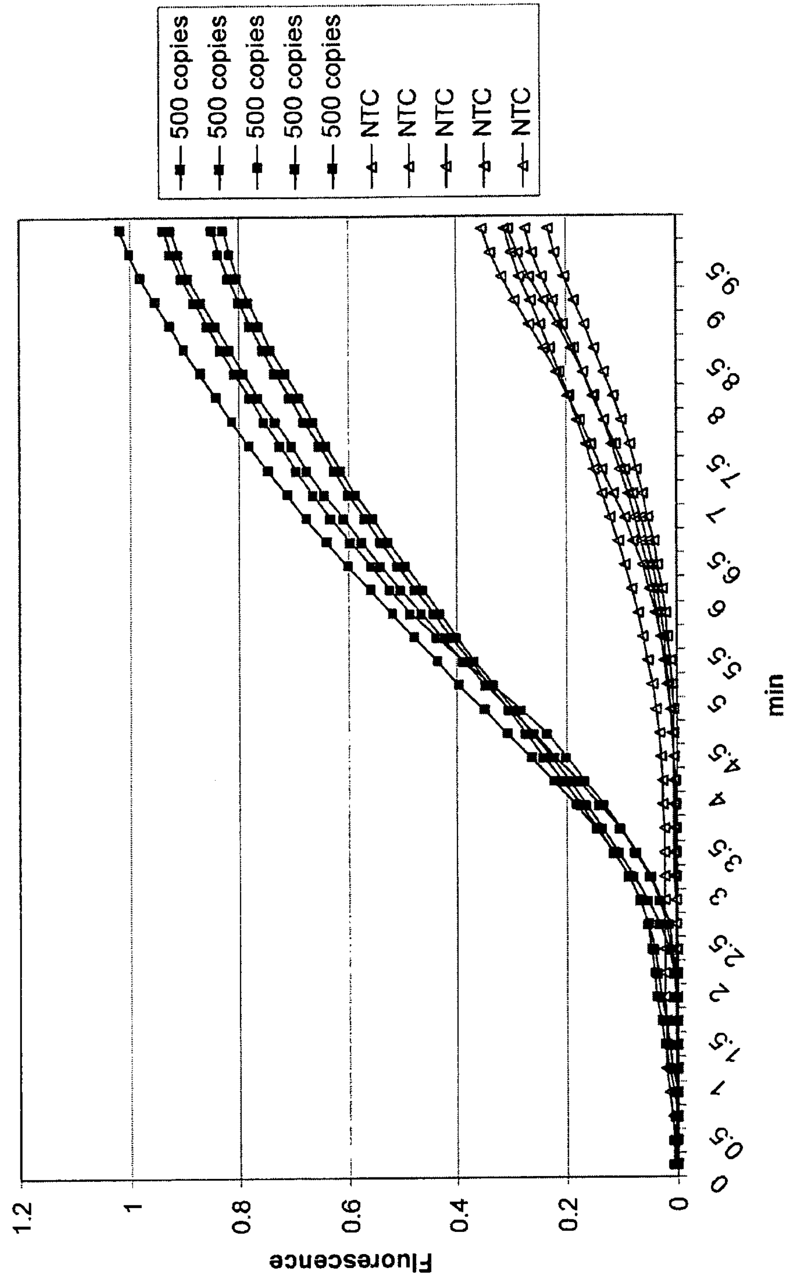


FIG. 6

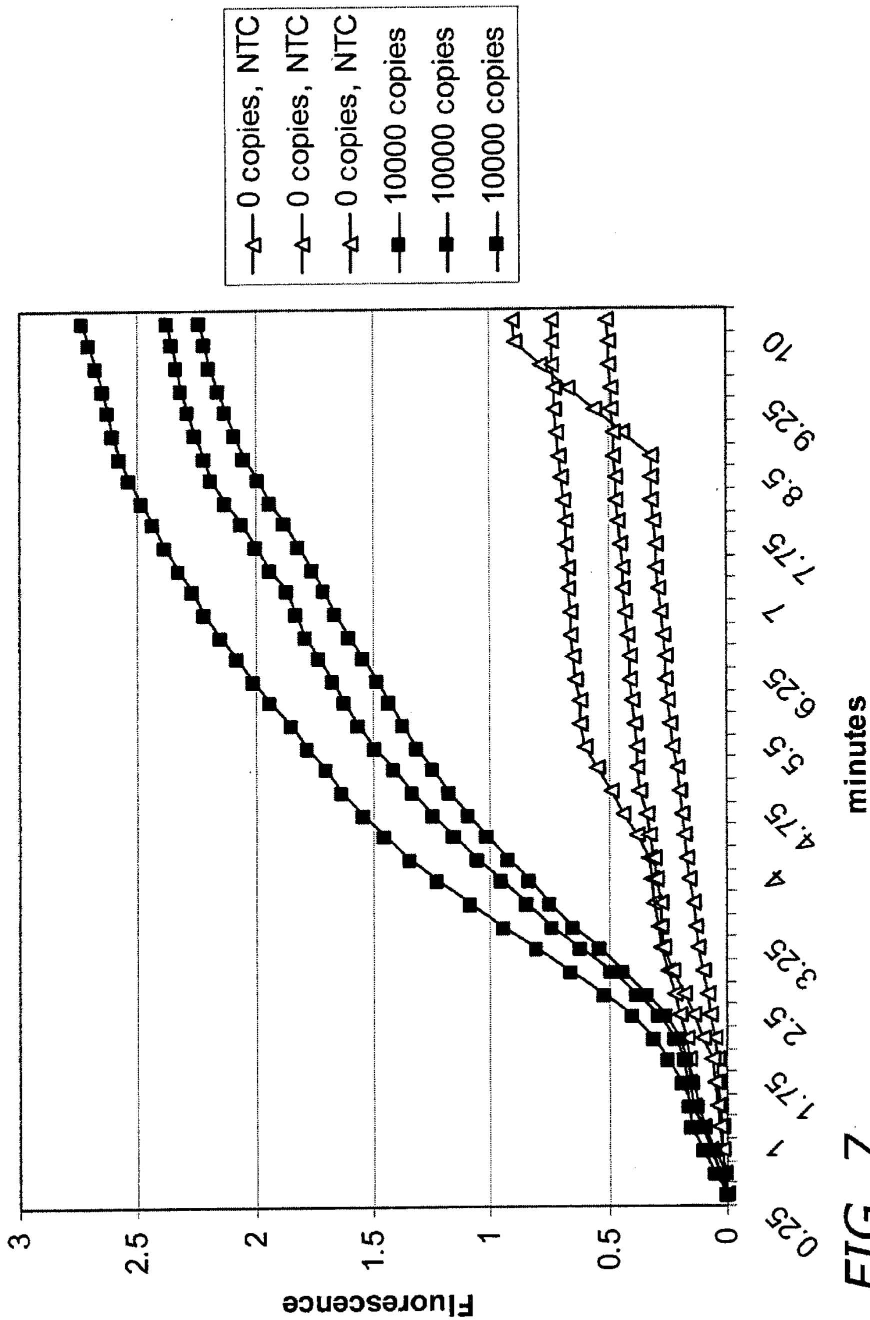


FIG. 7

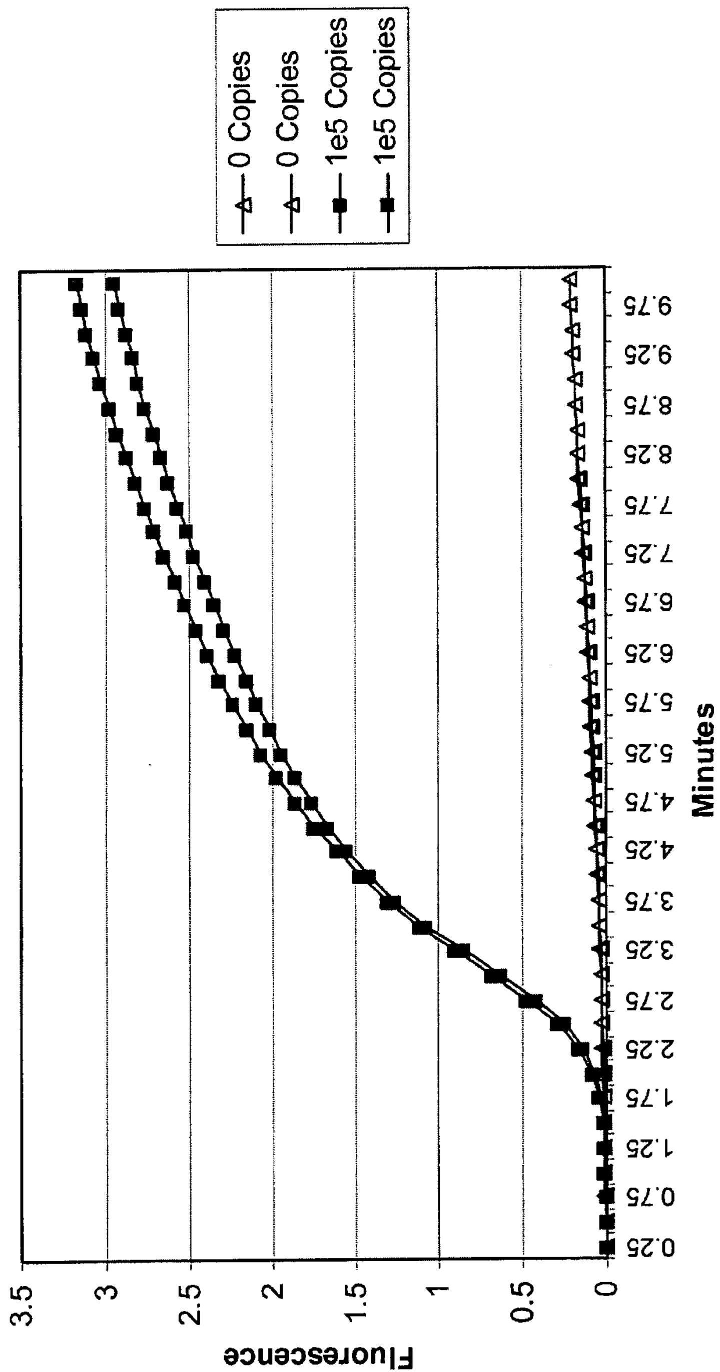


FIG. 8

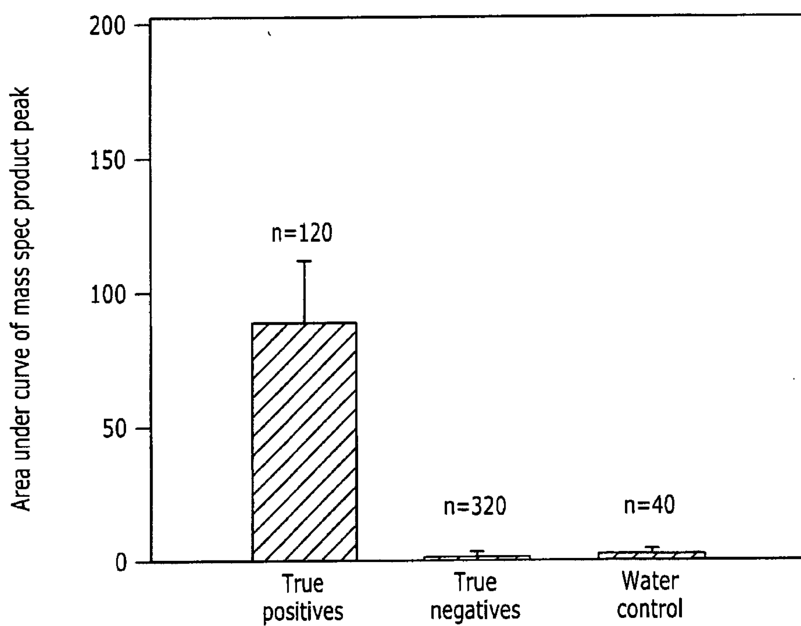


FIG. 9

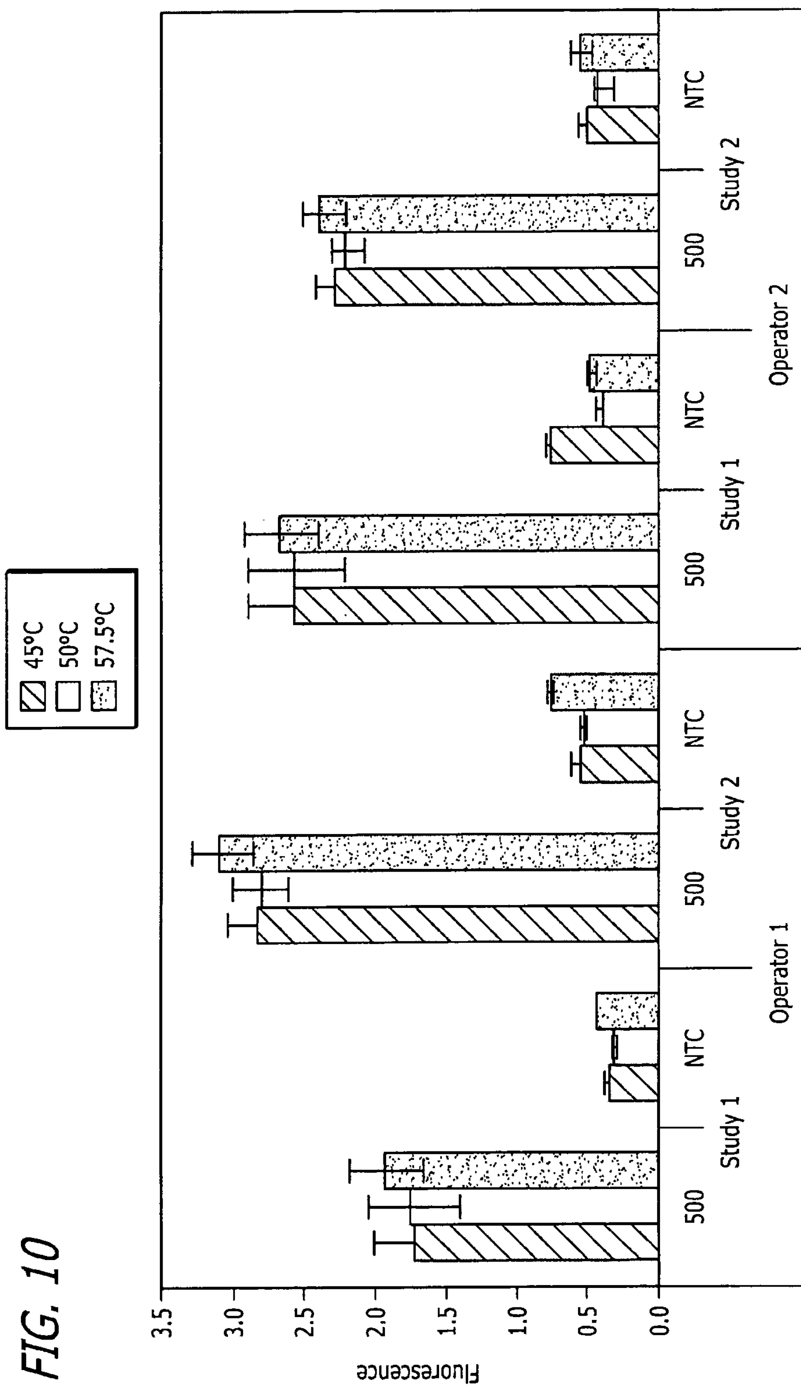


FIG. 10

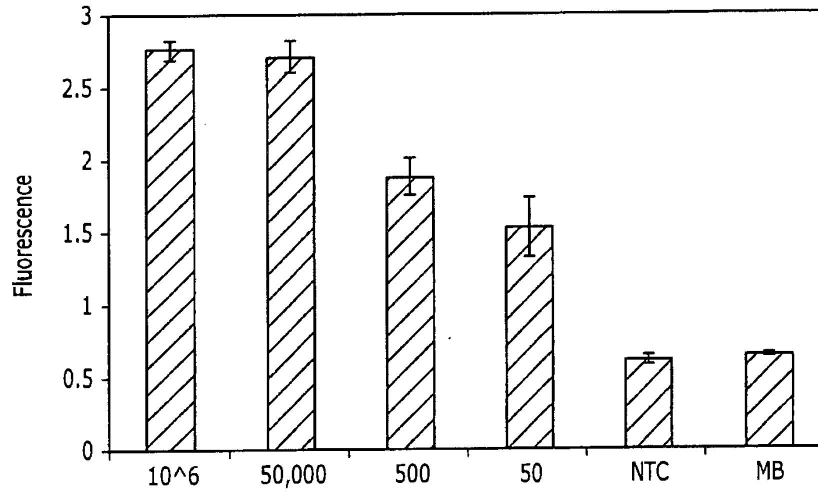


FIG. 11

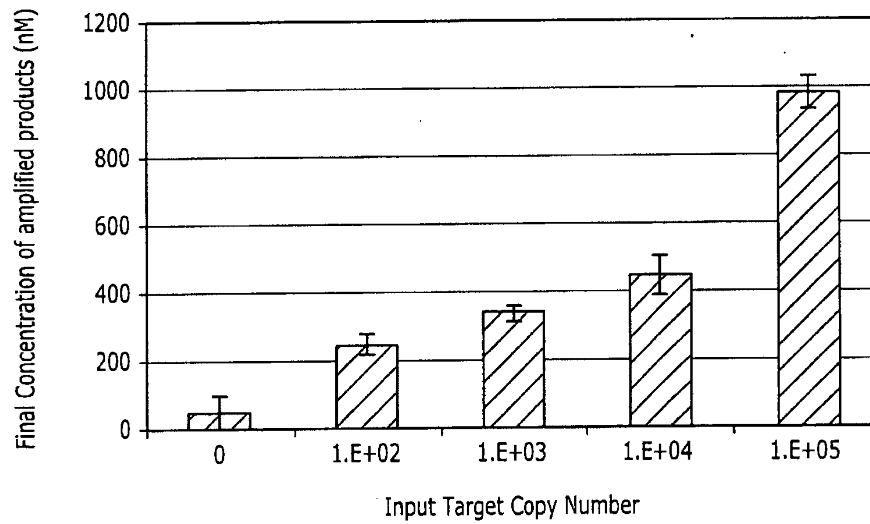


FIG. 12

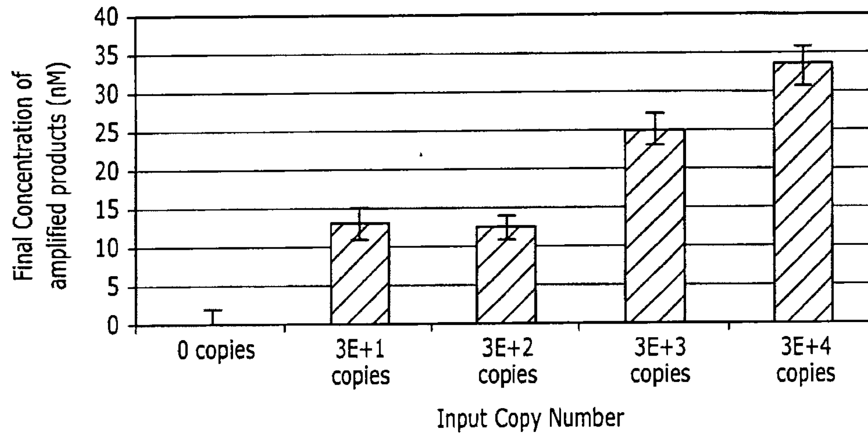


FIG. 13

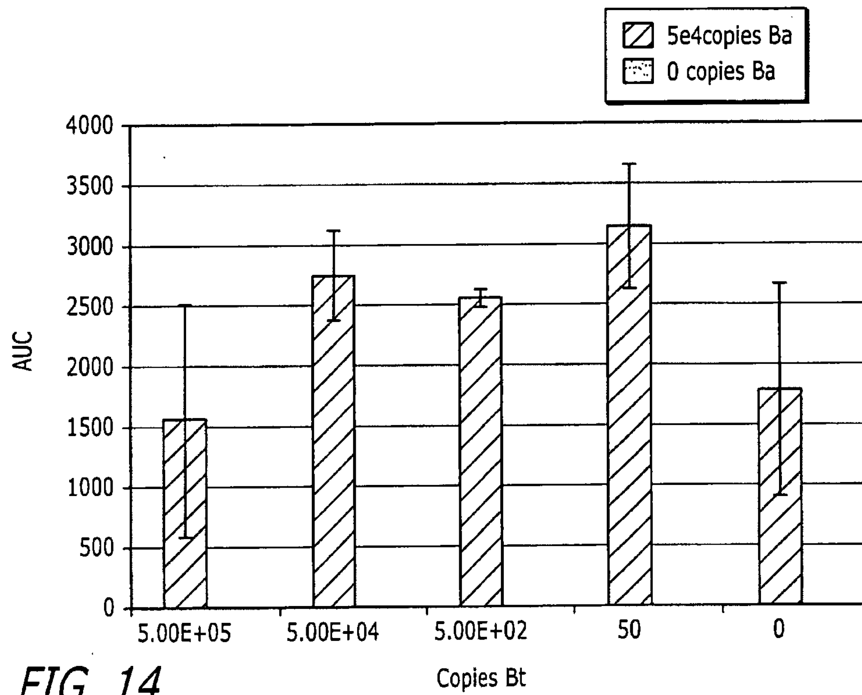


FIG. 14

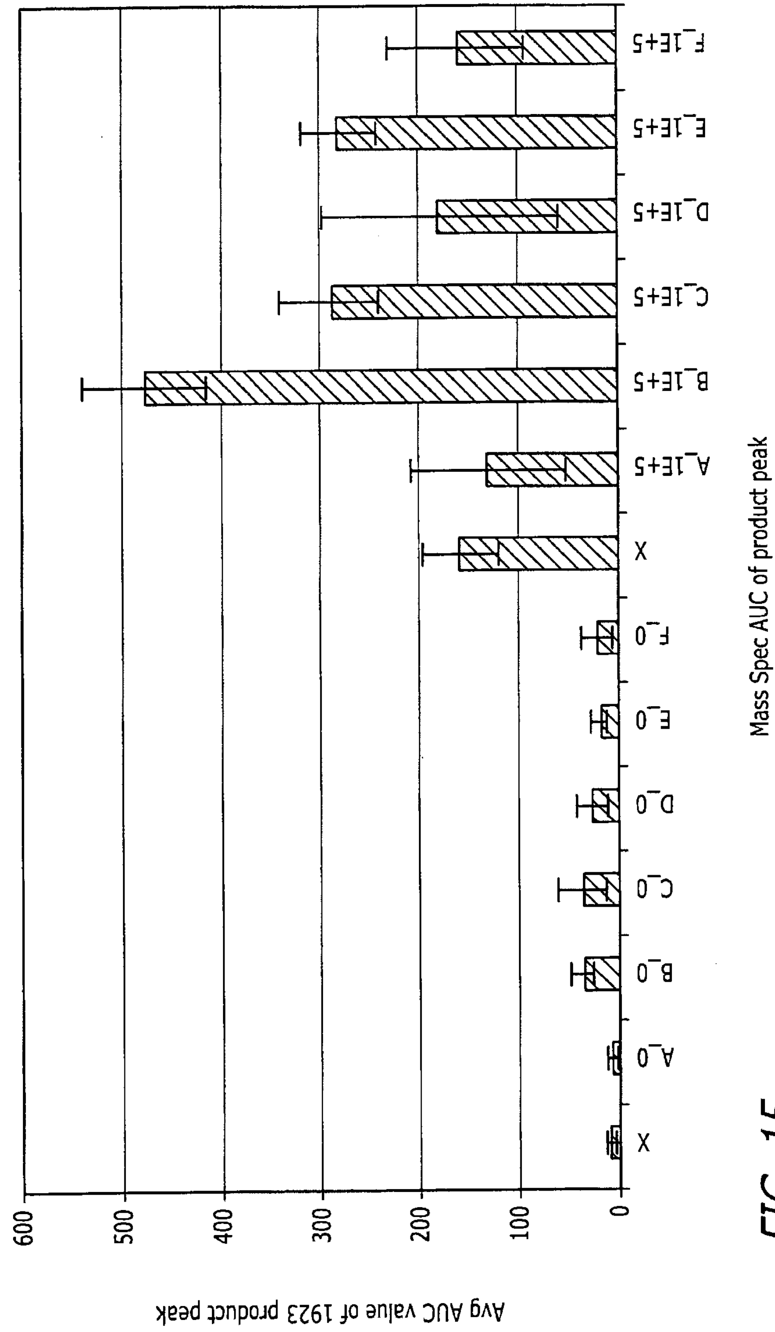


FIG. 15

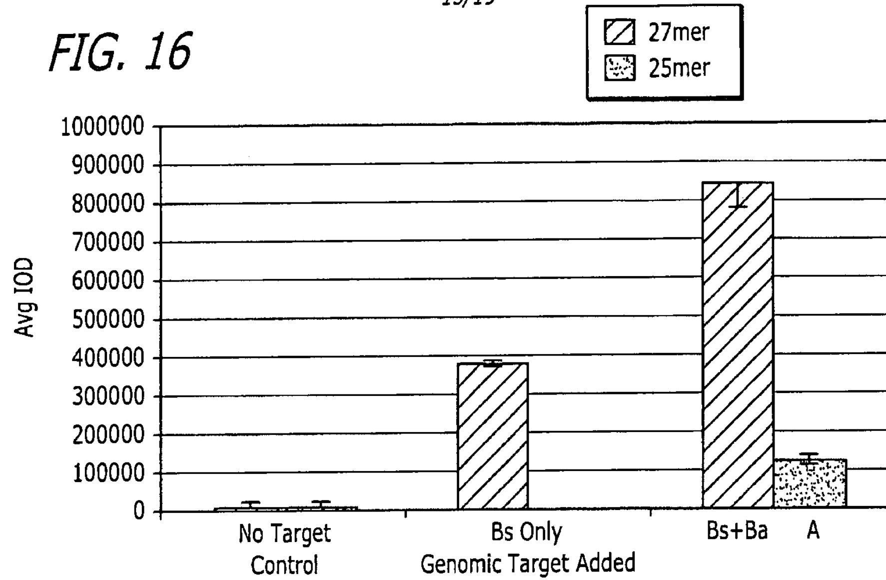


FIG. 16
27mer
25mer
1000000
900000
800000
700000
600000
500000
400000
300000
200000
100000
0
Avg IOD
No Target Control
Bs Only Genomic Target Added
Bs+Ba
A

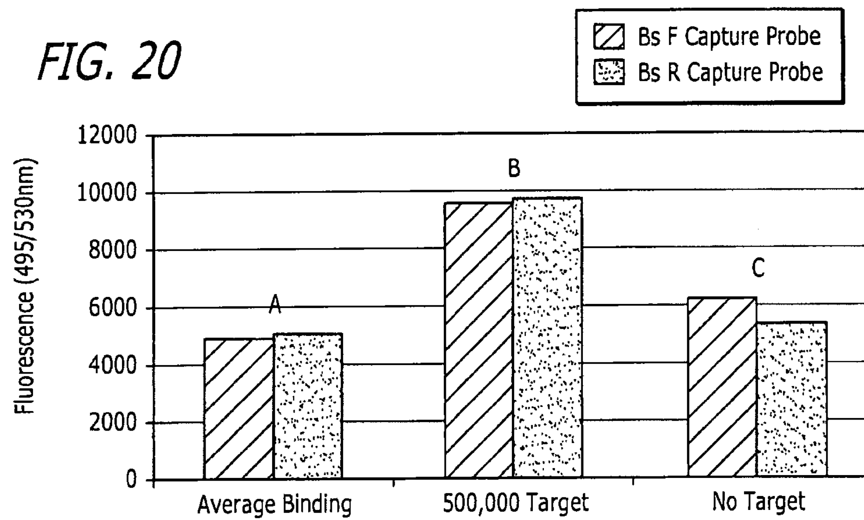


FIG. 20
Bs F Capture Probe
Bs R Capture Probe
12000
10000
8000
6000
4000
2000
0
Fluorescence (495/530nm)
A
B
C
Average Binding
500,000 Target
No Target

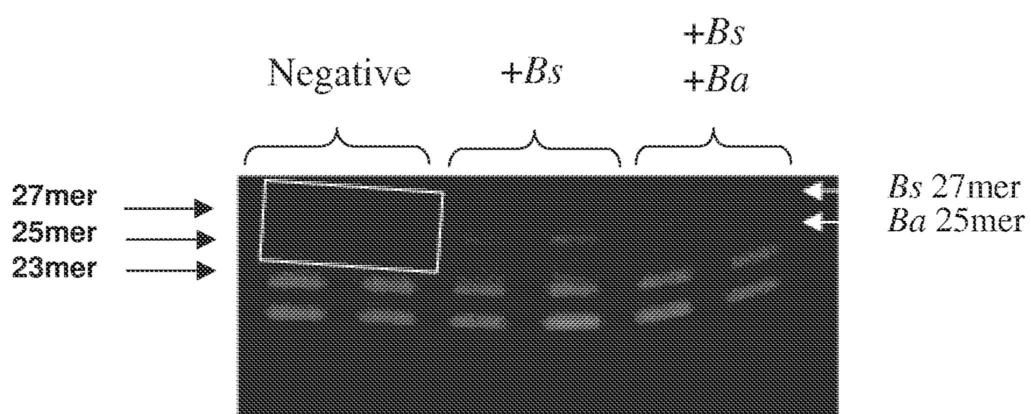


Figure 17

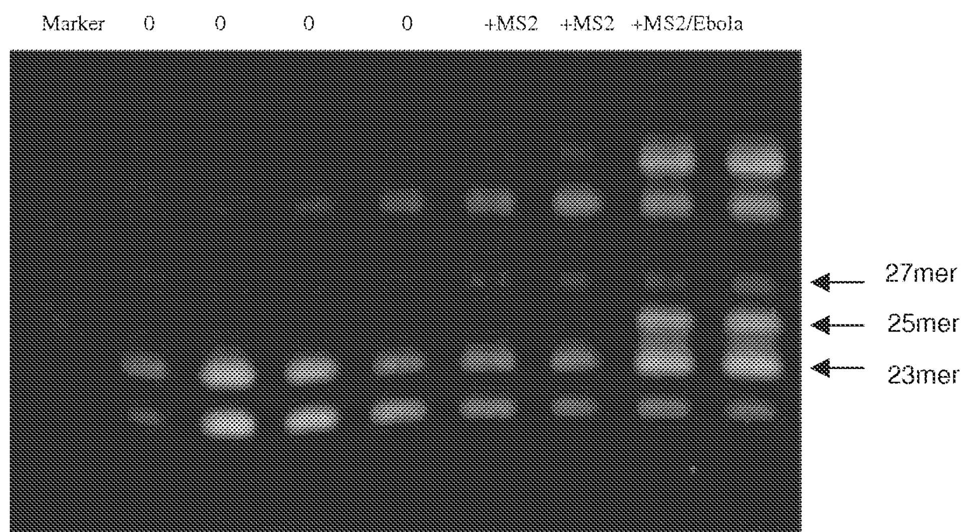
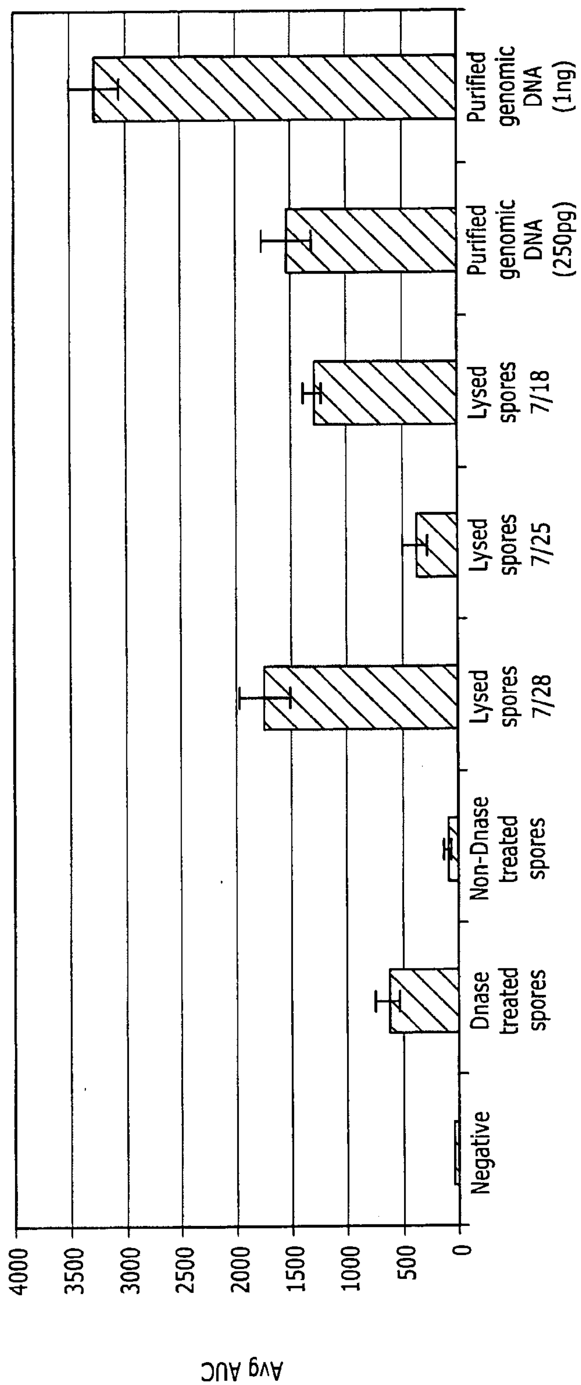


Figure 18

FIG. 19



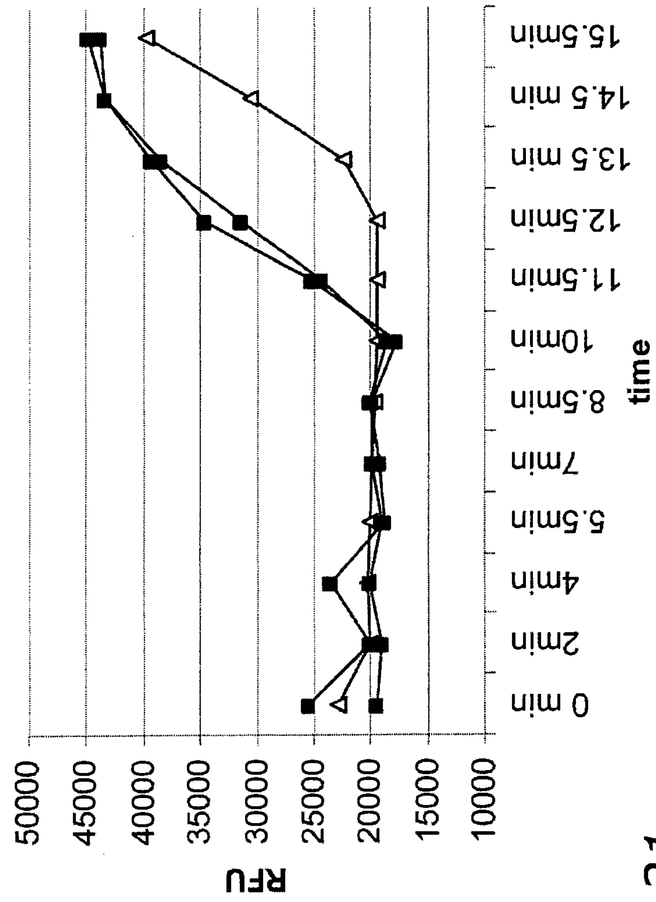


FIG. 21

Electronic Acknowledgement Receipt

EFS ID:	17591051
Application Number:	14067620
International Application Number:	
Confirmation Number:	4288
Title of Invention:	Nicking and Extension Amplification Reaction for the Exponential Amplification of Nucleic Acids
First Named Inventor/Applicant Name:	Brian K. Maples
Customer Number:	26161
Filer:	Ian J.S. Lodovice/Mary Florczak
Filer Authorized By:	Ian J.S. Lodovice
Attorney Docket Number:	30171-0025002
Receipt Date:	06-DEC-2013
Filing Date:	30-OCT-2013
Time Stamp:	15:42:03
Application Type:	Utility under 35 USC 111(a)

Payment information:

Submitted with Payment	no

File Listing:

Document Number	Document Description	File Name	File Size(Bytes)/ Message Digest	Multi Part /.zip	Pages (if appl.)
1	Applicant Response to Pre-Exam Formalities Notice	301710025002Response.pdf	53097 dc6695bed0b5088fccfafc29fba37fa34af3aa3f	no	1

Warnings:

Information:

2	Drawings-only black and white line drawings	23131301.pdf	4206166 51b0d6b0cc5bf07f41069ede605504d192a07948	no	19
Warnings:					
Information:					
		Total Files Size (in bytes):	4259263		

This Acknowledgement Receipt evidences receipt on the noted date by the USPTO of the indicated documents, characterized by the applicant, and including page counts, where applicable. It serves as evidence of receipt similar to a Post Card, as described in MPEP 503.

<u>New Applications Under 35 U.S.C. 111</u>
If a new application is being filed and the application includes the necessary components for a filing date (see 37 CFR 1.53(b)-(d) and MPEP 506), a Filing Receipt (37 CFR 1.54) will be issued in due course and the date shown on this Acknowledgement Receipt will establish the filing date of the application.

<u>National Stage of an International Application under 35 U.S.C. 371</u>
If a timely submission to enter the national stage of an international application is compliant with the conditions of 35 U.S.C. 371 and other applicable requirements a Form PCT/DO/EO/903 indicating acceptance of the application as a national stage submission under 35 U.S.C. 371 will be issued in addition to the Filing Receipt, in due course.

<u>New International Application Filed with the USPTO as a Receiving Office</u>
If a new international application is being filed and the international application includes the necessary components for an international filing date (see PCT Article 11 and MPEP 1810), a Notification of the International Application Number and of the International Filing Date (Form PCT/RO/105) will be issued in due course, subject to prescriptions concerning national security, and the date shown on this Acknowledgement Receipt will establish the international filing date of the application.

UNITED STATES PATENT AND TRADEMARK OFFICE

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APPLICATION NUMBER	FILING OR 371(C) DATE	FIRST NAMED APPLICANT	ATTY. DOCKET NO./TITLE
14/067,620	10/30/2013	Brian K. Maples	30171-0025002

CONFIRMATION NO. 4288

26161
FISH & RICHARDSON P.C. (BO)
P.O. BOX 1022
MINNEAPOLIS, MN 55440-1022

FORMALITIES LETTER

OC000000065232619

Date Mailed: 12/02/2013

NOTICE TO FILE CORRECTED APPLICATION PAPERS

Filing Date Granted

An application number and filing date have been accorded to this application. The application is informal since it does not comply with the regulations for the reason(s) indicated below. Applicant is given TWO MONTHS from the date of this Notice within which to correct the informalities indicated below. Extensions of time may be obtained by filing a petition accompanied by the extension fee under the provisions of 37 CFR 1.136(a).

The required item(s) identified below must be timely submitted to avoid abandonment:

- Replacement drawings in compliance with 37 CFR 1.84 and 37 CFR 1.121(d) are required. The drawings submitted are not acceptable because:
  - The drawings submitted to the Office are not electronically reproducible because portions of figures 5 are missing and/or blurry.

Applicant is cautioned that correction of the above items may cause the specification and drawings page count to exceed 100 pages. If the specification and drawings exceed 100 pages, applicant will need to submit the required application size fee.

Replies must be received in the USPTO within the set time period or must include a proper Certificate of Mailing or Transmission under 37 CFR 1.8 with a mailing or transmission date within the set time period. For more information and a suggested format, see Form PTO/SB/92 and MPEP 512.

Replies should be mailed to:

Mail Stop Missing Parts
Commissioner for Patents
P.O. Box 1450
Alexandria VA 22313-1450

Registered users of EFS-Web may alternatively submit their reply to this notice via EFS-Web.
https://sportal.uspto.gov/authenticate/AuthenticateUserLocalEPF.html

For more information about EFS-Web please call the USPTO Electronic Business Center at **1-866-217-9197** or visit our website at http://www.uspto.gov/ebc.

If you are not using EFS-Web to submit your reply, you must include a copy of this notice.

/ewondimu/

Office of Data Management, Application Assistance Unit (571) 272-4000, or (571) 272-4200, or 1-888-786-0101

PATENT APPLICATION FEE DETERMINATION RECORD

Substitute for Form PTO-875

Application or Docket Number: 14/067,620

APPLICATION AS FILED - PART I

FOR	NUMBER FILED (Column 1)		NUMBER EXTRA (Column 2)	SMALL ENTITY RATE($)	SMALL ENTITY FEE($)	OR	OTHER THAN SMALL ENTITY RATE($)	OTHER THAN SMALL ENTITY FEE($)
BASIC FEE (37 CFR 1.16(a), (b), or (c))	N/A		N/A	N/A			N/A	280
SEARCH FEE (37 CFR 1.16(k), (i), or (m))	N/A		N/A	N/A			N/A	600
EXAMINATION FEE (37 CFR 1.16(o), (p), or (q))	N/A		N/A	N/A			N/A	720
TOTAL CLAIMS (37 CFR 1.16(i))	29	minus 20 =	* 9			OR	x 80 =	720
INDEPENDENT CLAIMS (37 CFR 1.16(h))	3	minus 3 =	*				x 420 =	0.00
APPLICATION SIZE FEE (37 CFR 1.16(s))	If the specification and drawings exceed 100 sheets of paper, the application size fee due is $310 ($155 for small entity) for each additional 50 sheets or fraction thereof. See 35 U.S.C. 41(a)(1)(G) and 37 CFR 1.16(s).							0.00
MULTIPLE DEPENDENT CLAIM PRESENT (37 CFR 1.16(j))								0.00
				TOTAL			TOTAL	2320

* If the difference in column 1 is less than zero, enter "0" in column 2.

APPLICATION AS AMENDED - PART II

AMENDMENT A

	CLAIMS REMAINING AFTER AMENDMENT (Column 1)		HIGHEST NUMBER PREVIOUSLY PAID FOR (Column 2)	PRESENT EXTRA (Column 3)	SMALL ENTITY RATE($)	SMALL ENTITY ADDITIONAL FEE($)	OR	OTHER THAN SMALL ENTITY RATE($)	OTHER THAN SMALL ENTITY ADDITIONAL FEE($)
Total (37 CFR 1.16(i))	*	Minus	**	=	x =		OR	x =	
Independent (37 CFR 1.16(h))	*	Minus	***	=	x =		OR	x =	
Application Size Fee (37 CFR 1.16(s))									
FIRST PRESENTATION OF MULTIPLE DEPENDENT CLAIM (37 CFR 1.16(j))							OR		
					TOTAL ADD'L FEE		OR	TOTAL ADD'L FEE	

AMENDMENT B

	CLAIMS REMAINING AFTER AMENDMENT (Column 1)		HIGHEST NUMBER PREVIOUSLY PAID FOR (Column 2)	PRESENT EXTRA (Column 3)	SMALL ENTITY RATE($)	SMALL ENTITY ADDITIONAL FEE($)	OR	OTHER THAN SMALL ENTITY RATE($)	OTHER THAN SMALL ENTITY ADDITIONAL FEE($)
Total (37 CFR 1.16(i))	*	Minus	**	=	x =		OR	x =	
Independent (37 CFR 1.16(h))	*	Minus	***	=	x =		OR	x =	
Application Size Fee (37 CFR 1.16(s))									
FIRST PRESENTATION OF MULTIPLE DEPENDENT CLAIM (37 CFR 1.16(j))							OR		
					TOTAL ADD'L FEE		OR	TOTAL ADD'L FEE	

* If the entry in column 1 is less than the entry in column 2, write "0" in column 3.
** If the "Highest Number Previously Paid For" IN THIS SPACE is less than 20, enter "20".
*** If the "Highest Number Previously Paid For" IN THIS SPACE is less than 3, enter "3".
The "Highest Number Previously Paid For" (Total or Independent) is the highest found in the appropriate box in column 1.

UNITED STATES PATENT AND TRADEMARK OFFICE

UNITED STATES DEPARTMENT OF COMMERCE
United States Patent and Trademark Office
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P.O. Box 1450
Alexandria, Virginia 22313-1450
www.uspto.gov

APPLICATION NUMBER	FILING or 371(c) DATE	GRP ART UNIT	FIL FEE REC'D	ATTY.DOCKET.NO	TOT CLAIMS	IND CLAIMS
14/067,620	10/30/2013	1637	2620	30171-0025002	29	3

CONFIRMATION NO. 4288

FILING RECEIPT

26161
FISH & RICHARDSON P.C. (BO)
P.O. BOX 1022
MINNEAPOLIS, MN 55440-1022

Date Mailed: 12/02/2013

Receipt is acknowledged of this non-provisional patent application. The application will be taken up for examination in due course. Applicant will be notified as to the results of the examination. Any correspondence concerning the application must include the following identification information: the U.S. APPLICATION NUMBER, FILING DATE, NAME OF APPLICANT, and TITLE OF INVENTION. Fees transmitted by check or draft are subject to collection. Please verify the accuracy of the data presented on this receipt. **If an error is noted on this Filing Receipt, please submit a written request for a Filing Receipt Correction. Please provide a copy of this Filing Receipt with the changes noted thereon. If you received a "Notice to File Missing Parts" for this application, please submit any corrections to this Filing Receipt with your reply to the Notice. When the USPTO processes the reply to the Notice, the USPTO will generate another Filing Receipt incorporating the requested corrections**

Inventor(s)

Brian K. Maples, Lake Forest, CA;
Rebecca C. Holmberg, San Diego, CA;
Andrew P. Miller, San Diego, CA;
Jarrod Provins, Dana Point, CA;
Richard Roth, Carlsbad, CA;
Jeffrey Mandell, San Diego, CA;

Applicant(s)

Ionian Technologies, Inc., San Diego, CA

Assignment For Published Patent Application

Ionian Technologies, Inc., San Diego, CA

Power of Attorney: The patent practitioners associated with Customer Number 26161

Domestic Priority data as claimed by applicant

This application is a CON of 11/778,018 07/14/2007

Foreign Applications for which priority is claimed (You may be eligible to benefit from the **Patent Prosecution Highway** program at the USPTO. Please see http://www.uspto.gov for more information.) - None.
Foreign application information must be provided in an Application Data Sheet in order to constitute a claim to foreign priority. See 37 CFR 1.55 and 1.76.

If Required, Foreign Filing License Granted: 11/21/2013
The country code and number of your priority application, to be used for filing abroad under the Paris Convention, is **US 14/067,620**
Projected Publication Date: To Be Determined - pending completion of Corrected Papers

Non-Publication Request: No

Early Publication Request: No
Title

Nicking and Extension Amplification Reaction for the Exponential Amplification of Nucleic Acids

Preliminary Class

435

Statement under 37 CFR 1.55 or 1.78 for AIA (First Inventor to File) Transition Applications: No

PROTECTING YOUR INVENTION OUTSIDE THE UNITED STATES

Since the rights granted by a U.S. patent extend only throughout the territory of the United States and have no effect in a foreign country, an inventor who wishes patent protection in another country must apply for a patent in a specific country or in regional patent offices. Applicants may wish to consider the filing of an international application under the Patent Cooperation Treaty (PCT). An international (PCT) application generally has the same effect as a regular national patent application in each PCT-member country. The PCT process **simplifies** the filing of patent applications on the same invention in member countries, but **does not result** in a grant of "an international patent" and does not eliminate the need of applicants to file additional documents and fees in countries where patent protection is desired.

Almost every country has its own patent law, and a person desiring a patent in a particular country must make an application for patent in that country in accordance with its particular laws. Since the laws of many countries differ in various respects from the patent law of the United States, applicants are advised to seek guidance from specific foreign countries to ensure that patent rights are not lost prematurely.

Applicants also are advised that in the case of inventions made in the United States, the Director of the USPTO must issue a license before applicants can apply for a patent in a foreign country. The filing of a U.S. patent application serves as a request for a foreign filing license. The application's filing receipt contains further information and guidance as to the status of applicant's license for foreign filing.

Applicants may wish to consult the USPTO booklet, "General Information Concerning Patents" (specifically, the section entitled "Treaties and Foreign Patents") for more information on timeframes and deadlines for filing foreign patent applications. The guide is available either by contacting the USPTO Contact Center at 800-786-9199, or it can be viewed on the USPTO website at http://www.uspto.gov/web/offices/pac/doc/general/index.html.

For information on preventing theft of your intellectual property (patents, trademarks and copyrights), you may wish to consult the U.S. Government website, http://www.stopfakes.gov. Part of a Department of Commerce initiative, this website includes self-help "toolkits" giving innovators guidance on how to protect intellectual property in specific countries such as China, Korea and Mexico. For questions regarding patent enforcement issues, applicants may call the U.S. Government hotline at 1-866-999-HALT (1-866-999-4258).

LICENSE FOR FOREIGN FILING UNDER

Title 35, United States Code, Section 184

Title 37, Code of Federal Regulations, 5.11 & 5.15

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The applicant has been granted a license under 35 U.S.C. 184, if the phrase "IF REQUIRED, FOREIGN FILING LICENSE GRANTED" followed by a date appears on this form. Such licenses are issued in all applications where the conditions for issuance of a license have been met, regardless of whether or not a license may be required as set forth in 37 CFR 5.15. The scope and limitations of this license are set forth in 37 CFR 5.15(a) unless an earlier license has been issued under 37 CFR 5.15(b). The license is subject to revocation upon written notification. The date indicated is the effective date of the license, unless an earlier license of similar scope has been granted under 37 CFR 5.13 or 5.14.

This license is to be retained by the licensee and may be used at any time on or after the effective date thereof unless it is revoked. This license is automatically transferred to any related applications(s) filed under 37 CFR 1.53(d). This license is not retroactive.

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NOT GRANTED

No license under 35 U.S.C. 184 has been granted at this time, if the phrase "IF REQUIRED, FOREIGN FILING LICENSE GRANTED" DOES NOT appear on this form. Applicant may still petition for a license under 37 CFR 5.12, if a license is desired before the expiration of 6 months from the filing date of the application. If 6 months has lapsed from the filing date of this application and the licensee has not received any indication of a secrecy order under 35 U.S.C. 181, the licensee may foreign file the application pursuant to 37 CFR 5.15(b).

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UNITED STATES PATENT AND TRADEMARK OFFICE

UNITED STATES DEPARTMENT OF COMMERCE
United States Patent and Trademark Office
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Alexandria, Virginia 22313-1450
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APPLICATION NUMBER	FILING OR 371(C) DATE	FIRST NAMED APPLICANT	ATTY. DOCKET NO./TITLE
14/067,620	10/30/2013	Brian K. Maples	30171-0025002

CONFIRMATION NO. 4288

26161
FISH & RICHARDSON P.C. (BO)
P.O. BOX 1022
MINNEAPOLIS, MN 55440-1022

POA ACCEPTANCE LETTER

OC000000065196106

Date Mailed: 12/02/2013

NOTICE OF ACCEPTANCE OF POWER OF ATTORNEY

This is in response to the Power of Attorney filed 10/30/2013.

The Power of Attorney in this application is accepted. Correspondence in this application will be mailed to the above address as provided by 37 CFR 1.33.

/nmohammed/

Office of Data Management, Application Assistance Unit (571) 272-4000, or (571) 272-4200, or 1-888-786-0101

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Frederick P. Fish
1855-1930

W.K. Richardson
1859-1951

October 30, 2013

Attorney Docket No.: 30171-0025002/ITI-002 US

Commissioner for Patents
P.O. Box 1450
Alexandria, VA 22313-1450

Presented for filing is a new continuation patent application for prioritized examination of:

ATLANTA
AUSTIN
BOSTON
DALLAS
DELAWARE
HOUSTON
MUNICH
NEW YORK
SILICON VALLEY
SOUTHERN CALIFORNIA
TWIN CITIES
WASHINGTON, DC

Inventor(s): BRIAN K. MAPLES, REBECCA C. HOLMBERG, ANDREW P. MILLER, PH.D., JARROD PROVINS, RICHARD ROTH AND JEFFREY MANDELL

Title: NICKING AND EXTENSION AMPLIFICATION REACTION FOR THE EXPONENTIAL AMPLIFICATION OF NUCLEIC ACIDS

Enclosed are the following papers, including those required to receive a filing date under 37 C.F.R. § 1.53(b):

	Pages
Specification	44
Claims	7
Abstract	1
Declaration	6
Drawing(s)	24

Enclosures:

- — Application Data Sheet, 8 pages.
- — Preliminary amendment, 9 pages.
- — Power of Attorney, 1 page.
- — Statement under rule 3.73, 2 pages.
- — Certification and Request for Prioritized Examination (Track I), 1 page
- — Information Disclosure Statement, 1 page and Form PTO-1449, 8 pages

Basic Filing Fee	$280
Search Fee	$600

Examination Fee			$720
Publication fee			$300
Track I processing fee			$140
Track I prioritized examination fee			$4000
Total Claims 30	over 20	**10 x $80**	$800
Independent Claims 3	over 3	**0 x $420**	$0
Fee for Multiple Dependent claims			$0
Application size fee for each 50 pages over 100			
No (44 +22)*.75) - 100)/50 = No0 x			$0
Total Filing fee			$6840

The filing fee in the amount of $6840 is being paid concurrently herewith on the Electronic Filing System (EFS) by way of Deposit Account authorization. Please apply all charges or credits to Deposit Account No. 06-1050, referencing Attorney Docket No. 30171-0025002.

If this application is found to be incomplete, or if a telephone conference would otherwise be helpful, please call the undersigned at (617) 542-5070.

Please direct all correspondence to the following:

26161
PTO Customer Number

Respectfully submitted,

/Ian J.S. Lodovice, Reg. No. 59,749/

Ian J. Lodovice
Reg. No. 59,749
Enclosures
IJL/mkf
23103760.doc

Under the Paperwork Reduction Act of 1995, no persons are required to respond to a collection of information unless it contains a valid OMB control number.

Application Data Sheet 37 CFR 1.76	Attorney Docket Number	30171-0025002
	Application Number	

Title of Invention	Nicking and Extension Amplification Reaction for the Exponential Amplification of Nucleic Acids

The application data sheet is part of the provisional or nonprovisional application for which it is being submitted. The following form contains the bibliographic data arranged in a format specified by the United States Patent and Trademark Office as outlined in 37 CFR 1.76.
This document may be completed electronically and submitted to the Office in electronic format using the Electronic Filing System (EFS) or the document may be printed and included in a paper filed application.

Secrecy Order 37 CFR 5.2

☐ Portions or all of the application associated with this Application Data Sheet may fall under a Secrecy Order pursuant to 37 CFR 5.2 (Paper filers only. Applications that fall under Secrecy Order may not be filed electronically.)

Inventor Information:

Inventor 1 Remove
Legal Name

Prefix	Given Name	Middle Name	Family Name	Suffix
	Brian	K	Maples	

Residence Information (Select One) ◉ US Residency ◯ Non US Residency ◯ Active US Military Service

City	Lake Forest	State/Province	CA	Country of Residence i	US

Mailing Address of Inventor:

Address 1	4940 Carroll Canyon Road		
Address 2			
City	San Diego	State/Province	CA
Postal Code	92121	Country i	US

Inventor 2 Remove
Legal Name

Prefix	Given Name	Middle Name	Family Name	Suffix
	Rebecca	C.	Holmberg	

Residence Information (Select One) ◉ US Residency ◯ Non US Residency ◯ Active US Military Service

City		State/Province		Country of Residence i	

Mailing Address of Inventor:

Address 1	4940 Carroll Canyon Road		
Address 2			
City	San Diego	State/Province	CA
Postal Code	92121	Country i	US

Inventor 3 Remove
Legal Name

Prefix	Given Name	Middle Name	Family Name	Suffix
	Andrew	P.	Miller	

Residence Information (Select One) ◉ US Residency ◯ Non US Residency ◯ Active US Military Service

Application Data Sheet 37 CFR 1.76	Attorney Docket Number	30171-0025002
	Application Number	
Title of Invention	Nicking and Extension Amplification Reaction for the Exponential Amplification of Nucleic Acids	

City	San Diego	State/Province	CA	Country of Residence i	US

Mailing Address of Inventor:

Address 1	4940 Carroll Canyon Road		
Address 2			
City	San Diego	State/Province	CA
Postal Code	92121	Country i	US

Inventor 4 Remove
Legal Name

Prefix	Given Name	Middle Name	Family Name	Suffix
	Jarrod		Provins	

Residence Information (Select One) (●) US Residency ( ) Non US Residency ( ) Active US Military Service

City	Dana Point	State/Province	CA	Country of Residence i	US

Mailing Address of Inventor:

Address 1	4940 Carroll Canyon Road		
Address 2			
City	San Diego	State/Province	CA
Postal Code	92121	Country i	US

Inventor 5 Remove
Legal Name

Prefix	Given Name	Middle Name	Family Name	Suffix
	Richard		Roth	

Residence Information (Select One) (●) US Residency ( ) Non US Residency ( ) Active US Military Service

City	Carlsbad	State/Province	CA	Country of Residence i	US

Mailing Address of Inventor:

Address 1	4940 Carroll Canyon Road		
Address 2			
City	San Diego	State/Province	CA
Postal Code	92121	Country i	US

Inventor 6 Remove
Legal Name

Prefix	Given Name	Middle Name	Family Name	Suffix
	Jeffrey		Mandell	

Residence Information (Select One) (●) US Residency ( ) Non US Residency ( ) Active US Military Service

City		State/Province		Country of Residence i	

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Application Data Sheet 37 CFR 1.76	Attorney Docket Number	30171-0025002
	Application Number	
Title of Invention	Nicking and Extension Amplification Reaction for the Exponential Amplification of Nucleic Acids	

Mailing Address of Inventor:

Address 1	4940 Carroll Canyon Road		
Address 2			
City	San Diego	**State/Province**	CA
Postal Code	92121	**Country** i	US

All Inventors Must Be Listed - Additional Inventor Information blocks may be generated within this form by selecting the **Add** button. Add

Correspondence Information:

Enter either Customer Number or complete the Correspondence Information section below.
For further information see 37 CFR 1.33(a).

☐ **An Address is being provided for the correspondence Information of this application.**

Customer Number	26161
Email Address	Add Email / Remove Email

Application Information:

Title of the Invention	Nicking and Extension Amplification Reaction for the Exponential Amplification of Nucleic Acids		
Attorney Docket Number	30171-0025002	**Small Entity Status Claimed**	☐
Application Type	Nonprovisional		
Subject Matter	Utility		
Total Number of Drawing Sheets (if any)	24	**Suggested Figure for Publication (if any)**	

Publication Information:

☐ Request Early Publication (Fee required at time of Request 37 CFR 1.219)

☐ **Request Not to Publish.** I hereby request that the attached application not be published under 35 U.S.C. 122(b) and certify that the invention disclosed in the attached application **has not and will not** be the subject of an application filed in another country, or under a multilateral international agreement, that requires publication at eighteen months after filing.

Representative Information:

Representative information should be provided for all practitioners having a power of attorney in the application. Providing this information in the Application Data Sheet does not constitute a power of attorney in the application (see 37 CFR 1.32).
Either enter Customer Number or complete the Representative Name section below. If both sections are completed the customer Number will be used for the Representative Information during processing.

Please Select One:	◉ Customer Number	○ US Patent Practitioner	○ Limited Recognition (37 CFR 11.9)
Customer Number	26161		

Application Data Sheet 37 CFR 1.76	Attorney Docket Number	30171-0025002
	Application Number	

Title of Invention	Nicking and Extension Amplification Reaction for the Exponential Amplification of Nucleic Acids

Domestic Benefit/National Stage Information:

This section allows for the applicant to either claim benefit under 35 U.S.C. 119(e), 120, 121, or 365(c) or indicate National Stage entry from a PCT application. Providing this information in the application data sheet constitutes the specific reference required by 35 U.S.C. 119(e) or 120, and 37 CFR 1.78.

Prior Application Status	Pending		Remove
Application Number	Continuity Type	Prior Application Number	Filing Date (YYYY-MM-DD)
	Continuation of	11/778018	2007-07-14

Additional Domestic Benefit/National Stage Data may be generated within this form by selecting the **Add** button. Add

Foreign Priority Information:

This section allows for the applicant to claim priority to a foreign application. Providing this information in the application data sheet constitutes the claim for priority as required by 35 U.S.C. 119(b) and 37 CFR 1.55(d). When priority is claimed to a foreign application that is eligible for retrieval under the priority document exchange program (PDX) i the information will be used by the Office to automatically attempt retrieval pursuant to 37 CFR 1.55(h)(1) and (2). Under the PDX program, applicant bears the ultimate responsibility for ensuring that a copy of the foreign application is received by the Office from the participating foreign intellectual property office, or a certified copy of the foreign priority application is filed, within the time period specified in 37 CFR 1.55(g)(1).

			Remove
Application Number	Country i	Filing Date (YYYY-MM-DD)	Access Code i (if applicable)

Additional Foreign Priority Data may be generated within this form by selecting the **Add** button. Add

Statement under 37 CFR 1.55 or 1.78 for AIA (First Inventor to File) Transition Applications

☐ This application (1) claims priority to or the benefit of an application filed before March 16, 2013 and (2) also contains, or contained at any time, a claim to a claimed invention that has an effective filing date on or after March 16, 2013.
NOTE: By providing this statement under 37 CFR 1.55 or 1.78, this application, with a filing date on or after March 16, 2013, will be examined under the first inventor to file provisions of the AIA.

Under the Paperwork Reduction Act of 1995, no persons are required to respond to a collection of information unless it contains a valid OMB control number.

Application Data Sheet 37 CFR 1.76	Attorney Docket Number	30171-0025002
	Application Number	
Title of Invention	Nicking and Extension Amplification Reaction for the Exponential Amplification of Nucleic Acids	

Authorization to Permit Access:

☐ Authorization to Permit Access to the Instant Application by the Participating Offices

If checked, the undersigned hereby grants the USPTO authority to provide the European Patent Office (EPO), the Japan Patent Office (JPO), the Korean Intellectual Property Office (KIPO), the World Intellectual Property Office (WIPO), and any other intellectual property offices in which a foreign application claiming priority to the instant patent application is filed access to the instant patent application. See 37 CFR 1.14(c) and (h). This box should not be checked if the applicant does not wish the EPO, JPO, KIPO, WIPO, or other intellectual property office in which a foreign application claiming priority to the instant patent application is filed to have access to the instant patent application.

In accordance with 37 CFR 1.14(h)(3), access will be provided to a copy of the instant patent application with respect to: 1) the instant patent application-as-filed; 2) any foreign application to which the instant patent application claims priority under 35 U.S.C. 119(a)-(d) if a copy of the foreign application that satisfies the certified copy requirement of 37 CFR 1.55 has been filed in the instant patent application; and 3) any U.S. application-as-filed from which benefit is sought in the instant patent application.

In accordance with 37 CFR 1.14(c), access may be provided to information concerning the date of filing this Authorization.

Applicant Information:

Providing assignment information in this section does not substitute for compliance with any requirement of part 3 of Title 37 of CFR to have an assignment recorded by the Office.

Applicant 1 Remove

If the applicant is the inventor (or the remaining joint inventor or inventors under 37 CFR 1.45), this section should not be completed. The information to be provided in this section is the name and address of the legal representative who is the applicant under 37 CFR 1.43; or the name and address of the assignee, person to whom the inventor is under an obligation to assign the invention, or person who otherwise shows sufficient proprietary interest in the matter who is the applicant under 37 CFR 1.46. If the applicant is an applicant under 37 CFR 1.46 (assignee, person to whom the inventor is obligated to assign, or person who otherwise shows sufficient proprietary interest) together with one or more joint inventors, then the joint inventor or inventors who are also the applicant should be identified in this section.

Clear

◉ Assignee	○ Legal Representative under 35 U.S.C. 117	○ Joint Inventor
○ Person to whom the inventor is obligated to assign.	○ Person who shows sufficient proprietary interest	

If applicant is the legal representative, indicate the authority to file the patent application, the inventor is:

Name of the Deceased or Legally Incapacitated Inventor :	
If the Applicant is an Organization check here.	☒
Organization Name	Ionian Technologies, Inc.

Application Data Sheet 37 CFR 1.76	Attorney Docket Number	30171-0025002
	Application Number	

Title of Invention	Nicking and Extension Amplification Reaction for the Exponential Amplification of Nucleic Acids

Mailing Address Information:			
Address 1	4940 Carroll Canyon Road		
Address 2			
City	San Diego	**State/Province**	CA
Country i	US	Postal Code	92121
Phone Number		Fax Number	
Email Address			

Additional Applicant Data may be generated within this form by selecting the Add button. Add

Assignee Information including Non-Applicant Assignee Information:

Providing assignment information in this section does not subsitute for compliance with any requirement of part 3 of Title 37 of CFR to have an assignment recorded by the Office.

Assignee 1

Complete this section if assignee information, including non-applicant assignee information, is desired to be included on the patent application publication . An assignee-applicant identified in the "Applicant Information" section will appear on the patent application publication as an applicant. For an assignee-applicant, complete this section only if identification as an assignee is also desired on the patent application publication.

Remove

If the Assignee is an Organization check here. ☐

Prefix	**Given Name**	Middle Name	**Family Name**	Suffix

Mailing Address Information:			
Address 1			
Address 2			
City		**State/Province**	
Country i		Postal Code	
Phone Number		Fax Number	
Email Address			

Additional Assignee Data may be generated within this form by selecting the Add button. Add

Application Data Sheet 37 CFR 1.76	Attorney Docket Number	30171-0025002
	Application Number	
Title of Invention	Nicking and Extension Amplification Reaction for the Exponential Amplification of Nucleic Acids	

Signature:

Remove

NOTE: This form must be signed in accordance with 37 CFR 1.33. See 37 CFR 1.4 for signature requirements and certifications

Signature	/Ian J.S. Lodovice, Reg. No. 59,749/			Date (YYYY-MM-DD)	2013-10-30
First Name	Ian J.S.	Last Name	Lodovice	Registration Number	59749

Additional Signature may be generated within this form by selecting the Add button. Add

This collection of information is required by 37 CFR 1.76. The information is required to obtain or retain a benefit by the public which is to file (and by the USPTO to process) an application. Confidentiality is governed by 35 U.S.C. 122 and 37 CFR 1.14. This collection is estimated to take 23 minutes to complete, including gathering, preparing, and submitting the completed application data sheet form to the USPTO. Time will vary depending upon the individual case. Any comments on the amount of time you require to complete this form and/or suggestions for reducing this burden, should be sent to the Chief Information Officer, U.S. Patent and Trademark Office, U.S. Department of Commerce, P.O. Box 1450, Alexandria, VA 22313-1450. DO NOT SEND FEES OR COMPLETED FORMS TO THIS ADDRESS. **SEND TO: Commissioner for Patents, P.O. Box 1450, Alexandria, VA 22313-1450.**

Privacy Act Statement

The Privacy Act of 1974 (P.L. 93-579) requires that you be given certain information in connection with your submission of the attached form related to a patent application or patent. Accordingly, pursuant to the requirements of the Act, please be advised that: (1) the general authority for the collection of this information is 35 U.S.C. 2(b)(2); (2) furnishing of the information solicited is voluntary; and (3) the principal purpose for which the information is used by the U.S. Patent and Trademark Office is to process and/or examine your submission related to a patent application or patent. If you do not furnish the requested information, the U.S. Patent and Trademark Office may not be able to process and/or examine your submission, which may result in termination of proceedings or abandonment of the application or expiration of the patent.

The information provided by you in this form will be subject to the following routine uses:

1. The information on this form will be treated confidentially to the extent allowed under the Freedom of Information Act (5 U.S.C. 552) and the Privacy Act (5 U.S.C. 552a). Records from this system of records may be disclosed to the Department of Justice to determine whether the Freedom of Information Act requires disclosure of these records.
2. A record from this system of records may be disclosed, as a routine use, in the course of presenting evidence to a court, magistrate, or administrative tribunal, including disclosures to opposing counsel in the course of settlement negotiations.
3. A record in this system of records may be disclosed, as a routine use, to a Member of Congress submitting a request involving an individual, to whom the record pertains, when the individual has requested assistance from the Member with respect to the subject matter of the record.
4. A record in this system of records may be disclosed, as a routine use, to a contractor of the Agency having need for the information in order to perform a contract. Recipients of information shall be required to comply with the requirements of the Privacy Act of 1974, as amended, pursuant to 5 U.S.C. 552a(m).
5. A record related to an International Application filed under the Patent Cooperation Treaty in this system of records may be disclosed, as a routine use, to the International Bureau of the World Intellectual Property Organization, pursuant to the Patent Cooperation Treaty.
6. A record in this system of records may be disclosed, as a routine use, to another federal agency for purposes of National Security review (35 U.S.C. 181) and for review pursuant to the Atomic Energy Act (42 U.S.C. 218(c)).
7. A record from this system of records may be disclosed, as a routine use, to the Administrator, General Services, or his/her designee, during an inspection of records conducted by GSA as part of that agency's responsibility to recommend improvements in records management practices and programs, under authority of 44 U.S.C. 2904 and 2906. Such disclosure shall be made in accordance with the GSA regulations governing inspection of records for this purpose, and any other relevant (i.e., GSA or Commerce) directive. Such disclosure shall not be used to make determinations about individuals.
8. A record from this system of records may be disclosed, as a routine use, to the public after either publication of the application pursuant to 35 U.S.C. 122(b) or issuance of a patent pursuant to 35 U.S.C. 151. Further, a record may be disclosed, subject to the limitations of 37 CFR 1.14, as a routine use, to the public if the record was filed in an application which became abandoned or in which the proceedings were terminated and which application is referenced by either a published application, an application open to public inspections or an issued patent.
9. A record from this system of records may be disclosed, as a routine use, to a Federal, State, or local law enforcement agency, if the USPTO becomes aware of a violation or potential violation of law or regulation.

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

Applicant	:	Ionian Technologies Inc.	Art Unit	:	Unknown
Serial No.	:	Not Yet Assigned	Examiner	:	Unknown
Filed	:	Herewith			
Title	:	NICKING AND EXTENSION AMPLIFICATION REACTION FOR THE EXPONENTIAL AMPLIFICATION OF NUCLEIC ACIDS			

Commissioner for Patents
P.O. Box 1450
Alexandria, VA 22313-1450

PRELIMINARY AMENDMENT

Prior to examination, please amend the application as indicated on the following pages.

Amendments to the Specification:

Please add the following new paragraph after the title at page 1, line 4:

Related Applications

This application is a continuation of U.S. Application Serial No. 11/778,018, filed July 14, 2007, the entire contents of which are hereby incorporated.

Amendments to the Claims:

This listing of claims replaces all prior versions and listings of claims in the application:

Listing of Claims:

1. – 66. (Canceled)

67. (New) A method of amplifying, comprising:

preparing a mixture comprising:

(i) a target nucleic acid present in a sample obtained from an animal, the target nucleic acid having a target polynucleotide sequence,

(ii) a polymerase,

(iii) a nicking enzyme,

(iv) a first oligonucleotide comprising a nicking site and a nicking enzyme binding site, and

(v) a second oligonucleotide comprising a nicking site and a nicking enzyme binding site.

68. (New) The method of claim 67, wherein the target polynucleotide sequence is amplified from steps comprising:

(a) forming a first duplex comprising the target polynucleotide sequence and the first oligonucleotide;

(b) extending, using the polymerase, the first oligonucleotide along the target polynucleotide sequence to form an extended first oligonucleotide comprising a sequence complementary to the second oligonucleotide;

(c) forming a second duplex comprising the second oligonucleotide and the extended first oligonucleotide;

(d) extending, using the polymerase, the second oligonucleotide along the extended first oligonucleotide to form an extended second oligonucleotide comprising a sequence

complementary to the first oligonucleotide and a first double-stranded nicking enzyme binding site;

(e) nicking, with the nicking enzyme, at the nicking site on the first oligonucleotide to produce a first polynucleotide fragment;

(f) extending, using the polymerase, the first polynucleotide fragment along the extended second oligonucleotide to produce a double-stranded nucleic acid product and a second double-stranded nicking enzyme binding site.

69. (New) The method of claim 68, wherein the double-stranded nucleic acid product comprises:

i) a first strand and a second strand, wherein the first strand comprises a first polynucleotide sequence corresponding to the target polynucleotide sequence and the second strand comprises a second polynucleotide sequence complementary to the target polynucleotide sequence, and

ii) first and second double-stranded nicking sites spaced apart by the target polynucleotide sequence.

70. (New) The method of claim 68, further comprising the steps of:

a) nicking, using the nicking enzyme, the first nicking site of the double-stranded nucleic acid product to prepare a first polynucleotide fragment and nicking, using the nicking enzyme, the second nicking site of the double-stranded nucleic acid product to produce a second polynucleotide fragment;

b) extending, using the polymerase, a portion of the first polynucleotide fragment to produce a first product polynucleotide and extending, using a polymerase, a portion of the second polynucleotide fragment to produce a second product polynucleotide; and

c) nicking, using the nicking enzyme, the first product polynucleotide to release a copy of the first polynucleotide sequence and nicking, using the nicking enzyme, the second product polynucleotide to release a copy of the second polynucleotide sequence.

71. (New) The method of claim 67, wherein the animal is a human.

72. (New) The method of claim 67, wherein the target nucleic acid is obtained from an animal pathogen.

73. (New) The method of claim 72, wherein the animal pathogen is a single-stranded DNA virus, double-stranded DNA virus, or single-stranded RNA virus.

74. (New) The method of claim 72, wherein the animal pathogen is a bacterium.

75. (New) The method of claim 72, wherein the animal pathogen contains spores and the target polynucleotide is amplified from the spores without the need for lysis of the spores.

76. (New) The method of claim 67, wherein the sample obtained from an animal is obtained from the blood, bone marrow, mucus, lymph, hard tissues (e.g. liver, spleen, kidney, lung or ovary), biopsies, sputum, saliva, tears, faeces or urine of the animal.

77. (New) The method of claim 76, wherein the sample obtained from an animal is obtained from the mucus, sputum, or saliva of the animal.

78. (New) The method of claim 67, wherein the target nucleic acid is double-stranded DNA.

79. (New) The method of claim 67, wherein the target nucleic acid is single-stranded DNA.

80. (New) The method of claim 67, wherein the target nucleic acid is RNA.

81. (New) The method of claim 67, wherein the target nucleic acid is selected from the group consisting of genomic DNA, plasmid DNA, viral DNA, mitochondrial DNA, cDNA, synthetic double-stranded DNA and synthetic single-stranded DNA.

82. (New) The method of claim 81, wherein the target nucleic acid is genomic DNA.

83. (New) The method of claim 81, wherein the target nucleic acid is viral DNA.

84. (New) The method of claim 67, which is performed without an initial heat denaturation step.

85. (New) The method of claim 67, wherein the nicking enzyme is Nt.BstNBI.

86. (New) The method of claim 67, wherein the nicking enzyme does not nick within the target polynucleotide sequence.

87. (New) The method of claim 67, which is performed without the use of temperature cycling.

88. (New) The method of claim 67, which is performed at about 55°C-59°C.

89. (New) The method of claim 67, which is performed at a constant temperature for about 1 to 20 minutes.

90. (New) The method of claim 68, which is performed at a temperature higher than the melting temperature of the first oligonucleotide/target polynucleotide sequence complex.

91. (New) The method of claim 67, further comprising detecting amplification product.

92. (New) The method of claim 91, wherein the amplification product is detected by a detection method selected from the group consisting of gel electrophoresis, mass spectrometry, SYBR I fluorescence, SYBR II fluorescence, SYBR Gold, Pico Green, TOTO-3, intercalating dye detection, fluorescence resonance energy transfer (FRET), molecular beacon detection, surface capture, capillary electrophoresis, incorporation of labeled nucleotides to allow detection by capture, fluorescence polarization, and lateral flow capture, or a combination thereof.

93. (New) The method of claim 67, wherein the target polynucleotide sequence is amplified 1E+9-fold or more in about five minutes.

94. (New) A method of amplifying, comprising:

preparing a mixture comprising:

(i) a target nucleic acid present in a sample obtained from an animal, the target nucleic acid having a target polynucleotide sequence,

(ii) a polymerase,

(iii) a nicking enzyme,

(iv) a first oligonucleotide comprising a nicking site and a nicking enzyme binding site, and

(v) a second oligonucleotide comprising a nicking site and a nicking enzyme binding site; which method is performed without an initial heat denaturation step.

95. (New) A method of amplifying, comprising:

preparing a mixture comprising:

(i) genomic DNA present in a sample obtained from an animal, the genomic DNA having a target polynucleotide sequence,

(ii) a polymerase,

(iii) a nicking enzyme,

(iv) a first oligonucleotide comprising a nicking site and a nicking enzyme binding site, and

(v) a second oligonucleotide comprising a nicking site and a nicking enzyme binding site; which method is performed without an initial heat denaturation step.

REMARKS

Applicant respectfully request entry of the amendments submitted herein. Upon entry of the present amendment, claims 67-95 will be pending. Claims 1-66 are cancelled.

The specification is being amended to include a paragraph describing related applications, and claim the benefit of priority to such applications.

Please apply any other necessary charges or credits to Deposit Account 06-1050, referencing the above attorney docket number.

Respectfully submitted,

Date: October 30, 2013______________

/Ian J.S. Lodovice, Reg. No. 59,749/__________
Ian J. Lodovice
Reg. No. 59,749

Customer Number 26161
Fish & Richardson P.C.
Telephone: (617) 542-5070
Facsimile: (877) 769-7945

23103772.doc

Electronic Patent Application Fee Transmittal	
Application Number:	
Filing Date:	
Title of Invention:	Nicking and Extension Amplification Reaction for the Exponential Amplification of Nucleic Acids
First Named Inventor/Applicant Name:	Brian K. Maples
Filer:	Ian J.S. Lodovice/Mary Florczak
Attorney Docket Number:	30171-0025002
Filed as Large Entity	

Track I Prioritized Examination - Nonprovisional Application under 35 USC 111(a) Filing Fees

Description	Fee Code	Quantity	Amount	Sub-Total in USD($)
Basic Filing:				
Utility application filing	1011	1	280	280
Utility Search Fee	1111	1	600	600
Utility Examination Fee	1311	1	720	720
Request for Prioritized Examination	1817	1	4000	4000
Pages:				
Claims:				
Claims in Excess of 20	1202	10	80	800
Miscellaneous-Filing:				

Description	Fee Code	Quantity	Amount	Sub-Total in USD($)
Publ. Fee- Early, Voluntary, or Normal	1504	1	300	300
Petition:				
Patent-Appeals-and-Interference:				
Post-Allowance-and-Post-Issuance:				
Extension-of-Time:				
Miscellaneous:				
PROCESSING FEE, EXCEPT PROV. APPLS.	1830	1	140	140
		Total in USD ($)		**6840**

Electronic Acknowledgement Receipt

EFS ID:	17270612
Application Number:	14067620
International Application Number:	
Confirmation Number:	4288
Title of Invention:	Nicking and Extension Amplification Reaction for the Exponential Amplification of Nucleic Acids
First Named Inventor/Applicant Name:	Brian K. Maples
Customer Number:	26161
Filer:	Ian J.S. Lodovice/Renee Neuman
Filer Authorized By:	Ian J.S. Lodovice
Attorney Docket Number:	30171-0025002
Receipt Date:	30-OCT-2013
Filing Date:	
Time Stamp:	17:41:31
Application Type:	Utility under 35 USC 111(a)

Payment information:

Submitted with Payment	yes
Payment Type	Deposit Account
Payment was successfully received in RAM	$6840
RAM confirmation Number	5567
Deposit Account	061050
Authorized User	

File Listing:

Document Number	Document Description	File Name	File Size(Bytes)/ Message Digest	Multi Part /.zip	Pages (if appl.)

1	Power of Attorney	30171POA.pdf	311269 6c6f5ccbe08a2265012d6e4450684f46ffa5eeab	no	3
Warnings:					
Information:					
2	TrackOne Request	30171TrackRequest.pdf	138078 a97121849761b92f571ee37286e276ee4716f4c8	no	1
Warnings:					
Information:					
3	Information Disclosure Statement (IDS) Form (SB08)	30171IDS.pdf	536250 df16910c6e9add8ab916d9429554db4b2216a8e0	no	9
Warnings:					
Information:					
This is not an USPTO supplied IDS fillable form					
4		30171Application.pdf	3909287 b88fc2dd0315bfad675efc7a7702e376950e94e5	yes	76

Multipart Description/PDF files in .zip description

Document Description	Start	End
Specification	1	44
Claims	45	51
Abstract	52	52
Drawings-only black and white line drawings	53	76

Warnings:					
Information:					
5	Oath or Declaration filed	301710DEC.pdf	5700846 6a63dea9b0a54d06cd31cfc3ae3b48f672ce39a0	no	6
Warnings:					
Information:					
6	Transmittal of New Application	301710025002Transmittal.pdf	90627 17fa280a0fca40d1d4bc6fc5cc7c69c86ef777dc	no	3
Warnings:					
Information:					
7	Application Data Sheet	301710025002ADS.pdf	1509961 588c97bdc5f58cc529bf4841a048b3c557bf2e16	no	8

Warnings:

Information:

8		301710025002Prelim.pdf	112579 47da051400205c829352f0773a7458df252e0816	yes	8

Multipart Description/PDF files in .zip description

Document Description	Start	End
Preliminary Amendment	1	1
Specification	2	2
Claims	3	7
Applicant Arguments/Remarks Made in an Amendment	8	8

Warnings:

Information:

9	Fee Worksheet (SB06)	fee-info.pdf	42087 bc431bf06b83def009ff0dc2289f1da348477964	no	2

Warnings:

Information:

Total Files Size (in bytes):	12350984

This Acknowledgement Receipt evidences receipt on the noted date by the USPTO of the indicated documents, characterized by the applicant, and including page counts, where applicable. It serves as evidence of receipt similar to a Post Card, as described in MPEP 503.

New Applications Under 35 U.S.C. 111
If a new application is being filed and the application includes the necessary components for a filing date (see 37 CFR 1.53(b)-(d) and MPEP 506), a Filing Receipt (37 CFR 1.54) will be issued in due course and the date shown on this Acknowledgement Receipt will establish the filing date of the application.

National Stage of an International Application under 35 U.S.C. 371
If a timely submission to enter the national stage of an international application is compliant with the conditions of 35 U.S.C. 371 and other applicable requirements a Form PCT/DO/EO/903 indicating acceptance of the application as a national stage submission under 35 U.S.C. 371 will be issued in addition to the Filing Receipt, in due course.

New International Application Filed with the USPTO as a Receiving Office
If a new international application is being filed and the international application includes the necessary components for an international filing date (see PCT Article 11 and MPEP 1810), a Notification of the International Application Number and of the International Filing Date (Form PCT/RO/105) will be issued in due course, subject to prescriptions concerning national security, and the date shown on this Acknowledgement Receipt will establish the international filing date of the application.

STATEMENT UNDER 37 CFR 3.73(c)

Applicant/Patent Owner: Ionian Technologies Inc.

Application No./Patent No.: Not Yet Assigned Filed/Issue Date: Herewith

Titled: NICKING AND EXTENSION AMPLIFICATION REACTION FOR THE EXPONENTIAL AMPLIFICATION OF NUCLEIC ACIDS

Ionian Technologies, Inc., a corporation
(Name of Assignee) (Type of Assignee, e.g., corporation, partnership, university, government agency, etc.)

states that, for the patent application/patent identified above, it is (choose **one** of the option 1, 2, 3 or 4 below):

1. ☒ The assignee of the entire right, title, and interest.

2. ☐ An assignee of less than the entire right, title and interest (check applicable box):
   ☐ The extent (by percentage) of its ownership interest is _____. Additional Statement(s) by the owners holding the balance of the interest must be submitted to account for 100% of the ownership interest.

   ☐ There are unspecified percentages of ownership. The other parties, including inventors, who together own the entire right, title and interest are:

   Additional Statement(s) by the owner(s) holding the balance of the interest must be submitted to account for the entire right, title, and interest.

3. ☐ The assignee of an undivided interest in the entirety (a complete assignment from one of the joint inventors was made). The other parties, including inventors, who together own the entire right, title, and interest are:

   Additional Statement(s) by the owner(s) holding the balance of the interest must be submitted to account for the entire right, title, and interest.

4. ☐ The recipient, via a court proceeding or the like (e.g., bankruptcy, probate), of an undivided interest in the entirety (a complete transfer of ownership interest was made). The certified document(s) showing the transfer is attached.

The interest identified in option 1, 2 or 3 above (not option 4) is evidenced by either (choose **one** of the options A or B below):

A. ☒ An assignment from the inventor(s) of the patent application/patent identified above. The assignment was recorded in the United States Patent and Trademark Office at Reel 021171, Frame 0398.

B. ☐ A chain of title from the inventor(s), of the patent application/patent identified above, to the current assignee as follows:

   1. From: ______________________ To: ______________________
      The document was recorded in the United States Patent and Trademark Office at
      Reel ______, Frame ______, or for which a copy thereof is attached.

   2. From: ______________________ To: ______________________
      The document was recorded in the United States Patent and Trademark Office at
      Reel ______, Frame ______, or for which a copy thereof is attached.

This collection of information is required by 37 CFR 3.73(b). The information is required to obtain or retain a benefit by the public which is to file (and by the USPTO to process) an application. Confidentiality is governed by 35 U.S.C. 122 and 37 CFR 1.11 and 1.14. This collection is estimated to take 12 minutes to complete, including gathering, preparing, and submitting the completed application form to the USPTO. Time will vary depending upon the individual case. Any comments on the amount of time you require to complete this form and/or suggestions for reducing this burden, should be sent to the Chief Information Officer, U.S. Patent and Trademark Office, U.S. Department of Commerce, P.O. Box 1450, Alexandria, VA 22313-1450. DO NOT SEND FEES OR COMPLETED FORMS TO THIS ADDRESS. **SEND TO: Commissioner for Patents, P.O. Box 1450, Alexandria, VA 22313-1450.**

If you need assistance in completing the form, call 1-800-PTO-9199 and select option 2.

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STATEMENT UNDER 37 CFR 3.73(c)

3. From: ______________________________ To: ______________________________

The document was recorded in the United States Patent and Trademark Office at

Reel ______, Frame ______, or for which a copy thereof is attached.

4. From: ______________________________ To: ______________________________

The document was recorded in the United States Patent and Trademark Office at

Reel ______, Frame ______, or for which a copy thereof is attached.

5. From: ______________________________ To: ______________________________

The document was recorded in the United States Patent and Trademark Office at

Reel ______, Frame ______, or for which a copy thereof is attached.

6. From: ______________________________ To: ______________________________

The document was recorded in the United States Patent and Trademark Office at

Reel ______, Frame ______, or for which a copy thereof is attached.

☐ Additional documents in the chain of title are listed on a supplemental sheet(s).

☐ As required by 37 CFR 3.73(c)(1)(i), the documentary evidence of the chain of title from the original owner to the assignee was, or concurrently is being, submitted for recordation pursuant to 37 CFR 3.11.

[NOTE: A separate copy (*i.e.,* a true copy of the original assignment document(s)) must be submitted to Assignment Division in accordance with 37 CFR Part 3, to record the assignment in the records of the USPTO. See MPEP 302.08]

The undersigned (whose title is supplied below) is authorized to act on behalf of the assignee.

/Ian J.S. Lodovice, Reg. No.59,749/	October 30, 2013
Signature	Date
Ian J. Lodovice	59,749
Printed or Typed Name	Title or Registration Number

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PTO/AIA/80 (07-12)
Approved for use through 11/30/2014. OMB 0651-0035
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Under the Paperwork Reduction Act of 1995; no persons are required to respond to a collection of information unless it displays a valid OMB control number.

POWER OF ATTORNEY TO PROSECUTE APPLICATIONS BEFORE THE USPTO

I hereby revoke all previous powers of attorney given in the application identified in the attached statement under 37 CFR 3.73(c).

I hereby appoint:

☒ Practitioners associated with the Customer Number: 26161

OR

☐ Practitioner(s) named below (if more than ten patent practitioners are to be named, then a customer number must be used):

Name Registration	Number		Name Registration	Number

as attorney(s) or agent(s) to represent the undersigned before the United States Patent and Trademark Office (USPTO) in connection with any and all patent applications assigned only to the undersigned according to the USPTO assignment records or assignment documents attached to this form in accordance with 37 CFR 3.73(c).

Please change the correspondence address for the application identified in the attached statement under 37 CFR 3.73(c) to:

☒ The address associated with Customer Number: 26161

OR

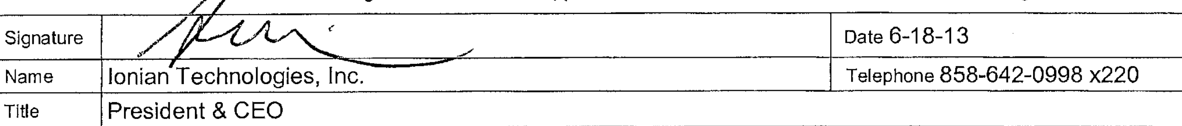
☐ Firm or Individual Name					
Address					
City		State		Zip	
Country					
Telephone		Email			

Assignee Name and Address:
Ionian Technologies, Inc.
4940 Carroll Canyon Road
San Diego, CA 92121

A copy of this form, together with a statement under 37 CFR 3.73(c) (Form PTO/SB/96 or equivalent) is required to be filed in each application in which this form is used. The statement under 37 CFR 3.73(c) may be completed by one of the practitioners appointed in this form, and must identify the application in which this Power of Attorney is to be filed.

SIGNATURE of Assignee of Record

The individual whose signature and title is supplied below is authorized to act on behalf of the assignee



Signature		Date 6-18-13
Name	Ionian Technologies, Inc.	Telephone 858-642-0998 x220
Title	President & CEO	

This collection of information is required by 37 CFR 1.31, 1.32 and 1.33. The information is required to obtain or retain a benefit by the public which is to file (and by the USPTO to process) an application. Confidentiality is governed by 35 U.S.C. 122 and 37 CFR 1.11 and 1.14. This collection is estimated to take 3 minutes to complete, including gathering, preparing, and submitting the completed application form to the USPTO. Time will vary depending upon the individual case. Any comments on the amount of time you require to complete this form and/or suggestions for reducing this burden, should be sent to the Chief Information Officer, U.S. Patent and Trademark Office, U.S. Department of Commerce, P.O. Box 1450, Alexandria, VA 22313-1450. DO NOT SEND FEES OR COMPLETED FORMS TO THIS ADDRESS. **SEND TO: Commissioner for Patents, P.O. Box 1450, Alexandria, VA 22313-1450.**

If you need assistance in completing the form, call 1-800-PTO-9199 and select option 2.

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CERTIFICATION AND REQUEST FOR PRIORITIZED EXAMINATION UNDER 37 CFR 1.102(e) (Page 1 of 1)

First Named Inventor:	Brian K. Maples	Nonprovisional Application Number (if known):	
Title of Invention:	Nicking and Extension Amplification Reaction for the Exponential Amplification of Nucleic Acids		

APPLICANT HEREBY CERTIFIES THE FOLLOWING AND REQUESTS PRIORITIZED EXAMINATION FOR THE ABOVE-IDENTIFIED APPLICATION.

1. The processing fee set forth in 37 CFR 1.17(i), the prioritized examination fee set forth in 37 CFR 1.17(c), and if not already paid, the publication fee set forth in 37 CFR 1.18(d) have been filed with the request. The basic filing fee, search fee, examination fee, and any required excess claims and application size fees are filed with the request or have been already been paid.
2. The application contains or is amended to contain no more than four independent claims and no more than thirty total claims, and no multiple dependent claims.
3. The applicable box is checked below:

I. ☒ Original Application (Track One) - Prioritized Examination under § 1.102(e)(1)

i. (a) The application is an original nonprovisional utility application filed under 35 U.S.C. 111(a). This certification and request is being filed with the utility application via EFS-Web.

---OR---

(b) The application is an original nonprovisional plant application filed under 35 U.S.C. 111(a). This certification and request is being filed with the plant application in paper.

ii. An executed oath or declaration under 37 CFR 1.63 is filed with the application.

II. ☐ Request for Continued Examination - Prioritized Examination under § 1.102(e)(2)

i. A request for continued examination has been filed with, or prior to, this form,
ii. If the application is a utility application, this certification and request is being filed via EFS-Web.
iii. The application is an original nonprovisional utility application filed under 35 U.S.C. 111(a), or is a national stage entry under 35 U.S.C. 371.
iv. This certification and request is being filed prior to the mailing of a first Office action responsive to the request for continued examination.
v. No prior request for continued examination has been granted prioritized examination status under 37 CFR 1.102(e)(2).

Signature /Ian J.S. Lodovice, Reg. No. 59,749/	Date October 30, 2013
Name Ian J. Lodovice (Print/Typed)	Practitioner Registration Number 59,749

***Note**: Signatures of all the inventors or assignees of record of the entire interest or their representative(s) are required in accordance with 37 CFR 1.33 and 11.18. Please see 37 CFR 1.4(d) for the form of the signature. If necessary, submit multiple forms for more than one signature, see below*.*

☒ *Total of 1 forms are submitted.

23103765.doc

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IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

Applicant : Ionian Technologies Inc. Art Unit : Unknown
Serial No. : Not Yet Assigned Examiner : Unknown
Filed : Herewith
Title : NICKING AND EXTENSION AMPLIFICATION REACTION FOR THE EXPONENTIAL AMPLIFICATION OF NUCLEIC ACIDS

Commissioner for Patents
P.O. Box 1450
Alexandria, VA 22313-1450

INFORMATION DISCLOSURE STATEMENT

Please consider the references listed on the enclosed PTO-SB-08 or Disclosure Form. Foreign patent documents and non-patent literature are enclosed; cited U.S. patents and patent application publications will be provided on request.

Under 35 USC §120, this application relies on the earlier filing date of application serial number 11/778,018, filed on July 14, 2007. The following references were submitted to and/or cited by the Office in the prior application and, therefore, are not provided in this application.

This statement is being filed with the application. Please apply any necessary charges or credits to Deposit Account 06-1050, referencing the above attorney docket number.

Respectfully submitted,

Date: October 30, 2013______________

/Ian J.S. Lodovice, Reg. No. 59,749/__________
Ian J. Lodovice
Reg. No. 59,749

Customer Number 26161
Fish & Richardson P.C.
Telephone: (617) 956-5972
Facsimile: (877) 769-7945

23103782.doc

Substitute Disclosure Form	U.S. Department of Commerce Patent and Trademark Office	Attorney Docket No. 30171-0025002	Application No. Not Yet Assigned
Information Disclosure Statement by Applicant (Use several sheets if necessary)		Applicant Ionian Technologies Inc.	
(37 CFR §1.98(b))		Filing Date Herewith	Group Art Unit Unknown

U.S. Patent Documents

Examiner Initial	Desig. ID	Document Number	Publication Date	Patentee	Class	Subclass	Filing Date If Appropriate
	1	5,210,015	5/11/1993	Gelfand et al.			
	2	5,270,184	12/14/1993	Walker et al.			
	3	5,397,698	3/19/1995	Goodman et al.			
	4	5,487,972	1/30/1996	Gelfand et al.			
	5	5,747,246	5/5/1998	Pannetier et al.			
	6	5,747,255	5/5/1998	Brenner			
	7	5,804,375	9/8/1998	Gelfand et al.			
	8	5,846,717	12/8/1998	Brow et al.			
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Substitute Disclosure Form	U.S. Department of Commerce Patent and Trademark Office	Attorney Docket No. 30171-0025002	Application No. Not Yet Assigned
Information Disclosure Statement by Applicant (Use several sheets if necessary)		Applicant Ionian Technologies Inc.	
(37 CFR §1.98(b))		Filing Date Herewith	Group Art Unit Unknown

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INFORMATION DISCLOSURE STATEMENT BY APPLICANT
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Complete if Known	
Application Number	11/778,018
Filing Date	July 14, 2007
First Named Inventor	MAPLES, Brian K.
Group Art Unit	1635
Examiner Name	NOT YET ASSIGNED
Attorney Docket No: ITI-1001-UT	

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NICKING AND EXTENSION AMPLIFICATION REACTION FOR THE EXPONENTIAL AMPLIFICATION OF NUCLEIC ACIDS

Field of the Invention

5 The invention is in general directed to the rapid exponential amplification of short DNA or RNA sequences at a constant temperature.

Background

The field of *in vitro* diagnostics is quickly expanding as the need for systems that can 10 rapidly detect the presence of harmful species or determine the genetic sequence of a region of interest is increasing exponentially. Current molecular diagnostics focus on the detection of biomarkers and include small molecule detection, immuno-based assays, and nucleic acid tests. The built-in specificity between two complementary nucleic acid strands allows for fast and specific recognition using unique DNA or RNA sequences, the simplicity of which makes a 15 nucleic acid test an attractive prospect. Identification of bacterial and viral threat agents, genetically modified food products, and single nucleotide polymorphisms for disease management are only a few areas where the advancement of these molecular diagnostic tools becomes extremely advantageous. To meet these growing needs, nucleic acid amplification technologies have been developed and tailored to these needs of specificity and sensitivity.

20 Historically, the most common amplification technique is the polymerase chain reaction (PCR), which has in many cases become the gold standard for detection methods because of its reliability and specificity. This technique requires the cycling of temperatures to proceed through the steps of denaturation of the dsDNA, annealing of short oligonucleotide primers, and extension of the primer along the template by a thermostable polymerase. Though many new 25 advances in engineering have successfully shortened these reaction times to 20-30 minutes, there is still a steep power requirement to meet the needs of these thermocycling units.

Various isothermal amplification techniques have been developed to circumvent the need for temperature cycling. From this demand, both DNA and RNA isothermal amplification technologies have emerged.

30 Transcription-Mediated Amplification (TMA) employs a reverse transcriptase with RNase activity, an RNA polymerase, and primers with a promoter sequence at the 5′end. The

reverse transcriptase synthesizes cDNA from the primer, degrades the RNA target, and synthesizes the second strand after the reverse primer binds. RNA polymerase then binds to the promoter region of the dsDNA and transcribes new RNA transcripts which can serve as templates for further reverse transcription. The reaction can produce a billion fold amplification in 20-30 minutes. This system is not as robust as other DNA amplification techniques and is therefore, not a field-deployable test due to the ubiquitous presence of RNAases outside of a sterile laboratory. This amplification technique is very similar to Self-Sustained Sequence Replication (3SR) and Nucleic Acid Sequence Based Amplification (NASBA), but varies in the enzymes employed.

Single Primer Isothermal Amplification (SPIA) also involves multiple polymerases and RNaseH. First, a reverse transcriptase extends a chimeric primer along an RNA target. RNaseH degrades the RNA target and allows a DNA polymerase to synthesize the second strand of cDNA. RNaseH then degrades a portion of the chimeric primer to release a portion of the cDNA and open a binding site for the next chimeric primer to bind and the amplification process proceeds through the cycle again. The linear amplification system can amplify very low levels of RNA target in roughly 3.5 hrs.

The Q-Beta replicase system is a probe amplification method. A probe region complementary to the target of choice is inserted into MDV-1 RNA, a naturally occurring template for Q-Beta replicase. Q-Beta replicates the MDV-1 plasmid so that the synthesized product is itself a template for Q-Beta replicase, resulting in exponential amplification as long as the there is excess replicase to template. Because the Q-Beta replication process is so sensitive and can amplify whether the target is present or not, multiple wash steps are required to purge the sample of non-specifically bound replication plasmids. The exponential amplification takes approximately 30 minutes; however, the total time including all wash steps is approximately 4 hours.

Numerous isothermal DNA amplification technologies have been developed as well. Rolling circle amplification (RCA) was developed based on the natural replication of plasmids and viruses. A primer extends along a circular template resulting in the synthesis of a single-stranded tandem repeat. Capture, washing, and ligation steps are necessary to preferentially circularize the template in the presence of target and reduce background amplification. Ramification amplification (RAM) adds cascading primers for additional geometric

amplification. This technique involves amplification of non-specifically sized strands that are either double or single-stranded.

Helicase-dependent amplification (HDA) takes advantage of a thermostable helicase (Tte-UvrD) to unwind dsDNA to create single-strands that are then available for hybridization
5 and extension of primers by polymerase. The thermostable HDA method does not require the accessory proteins that the non-thermostable HDA requires. The reaction can be performed at a single temperature, though an initial heat denaturation to bind the primers generates more product. Reaction times are reported to be over 1 hour to amplify products 70-120 base pairs in length.

10 Loop mediated amplification (LAMP) is a sensitive and specific isothermal amplification method that employs a thermostable polymerase with strand displacement capabilities and four or more primers. The primers are designed to anneal consecutively along the target in the forward and reverse direction. Extension of the outer primers displaces the extended inner primers to release single strands. Each primer is designed to have hairpin ends that, once
15 displaced, snap into a hairpin to facilitate self-priming and further polymerase extension. Additional loop primers can decrease the amplification time, but complicates the reaction mixture. Overall, LAMP is a difficult amplification method to multiplex, that is, to amplify more than one target sequence at a time, although it is reported to be extremely specific due to the multiple primers that must anneal to the target to further the amplification process. Though the
20 reaction proceeds under isothermal conditions, an initial heat denaturation step is required for double-stranded targets. Amplification proceeds in 25 to 50 minutes and yields a ladder pattern of various length products.

Strand displacement amplification (SDA) was developed by Walker et.al. in 1992. This amplification method uses two sets of primers, a strand displacing polymerase, and a restriction
25 endonuclease. The bumper primers serve to displace the initially extended primers to create a single-strand for the next primer to bind. A restriction site is present in the 5′ region of the primer. Thiol-modified nucleotides are incorporated into the synthesized products to inhibit cleavage of the synthesized strand. This modification creates a nick site on the primer side of the strand, which the polymerase can extend. This approach requires an initial heat denaturation step
30 for double-stranded targets. The reaction is then run at a temperature below the melting

temperature of the double-stranded target region. Products 60 to 100 bases in length are usually amplified in 30-45 minutes using this method.

These and other amplification methods are discussed in, for example, VanNess, J, et al., PNAS 2003. vol 100, no 8, p 4504-4509; Tan, E., et al., Anal. Chem. 2005, 77, 7984-7992;
5 Lizard, P., et al., Nature Biotech. 1998, 6, 1197-1202; Notomi, T., et al., NAR 2000, 28, 12, e63; and Kurn, N., et al., Clin. Chem. 2005, 51:10, 1973-1981. Other references for these general amplification techniques include, for example, U.S. Patent Serial Nos. 7112423; 5455166; 5712124; 5744311; 5916779; 5556751; 5733733; 5834202; 5354668; 5591609; 5614389; 5942391; and U.S. patent publication numbers US20030082590; US20030138800;
10 US20040058378; and US20060154286.

There is a need for a quicker method of amplification of single-stranded and double-stranded nucleic acid target sequences that can be performed without temperature cycling and that is suitable for shorter target sequences.

15 Summary

Provided herein are methods of amplifying nucleic acid target sequences that rely on nicking and extension reactions and amplify shorter sequences in a quicker timeframe than traditional amplification reactions, such as, for example, strand displacement amplification reactions. Embodiments of the invention include, for example, reactions that use only two
20 templates to prime, one or two nicking enzymes, and a polymerase, under isothermal conditions. In exemplary embodiments, the polymerase and the nicking enzyme are thermophilic, and the reaction temperature is significantly above the melting temperature of the hybridized target region. The nicking enzyme nicks only one strand in a double-stranded duplex, so that incorporation of modified nucleotides is not necessary as it is in strand displacement. An initial
25 heat denaturation step is not required for the methods of the present invention. Due to the simplicity of the reaction, in exemplary embodiments, the reaction is very easy to perform and can amplify 20-30mer products 10^8 to 10^{10} fold from genomic DNA in 2.5 to 10 minutes. Furthermore, in other exemplary embodiments, the method is able to amplify RNA without a separate reverse transcription step.

30 Thus, provided in a first embodiment of the present invention is a method for amplifying a double-stranded nucleic acid target sequence, comprising contacting a target DNA molecule

comprising a double-stranded target sequence having a sense strand and an antisense strand, with a forward template and a reverse template, wherein said forward template comprises a nucleic acid sequence comprising a recognition region at the 3′ end that is complementary to the 3′ end of the target sequence antisense strand; a nicking enzyme site upstream of said recognition
5 region, and a stabilizing region upstream of said nicking enzyme site; said reverse template comprises a nucleotide sequence comprising a recognition region at the 3′ end that is complementary to the 3′ end of the target sequence sense strand, a nicking enzyme site upstream of said recognition region, and a stabilizing region upstream of said nicking enzyme site; providing a first nicking enzyme that is capable of nicking at the nicking enzyme site of said
10 forward template, and does not nick within said target sequence; providing a second nicking enzyme that is capable of nicking at the nicking enzyme site of said reverse template and does not nick within said target sequence; and providing a DNA polymerase; under conditions wherein amplification is performed by multiple cycles of said polymerase extending said forward and reverse templates along said target sequence producing a double-stranded nicking enzyme
15 site, and said nicking enzymes nicking at said nicking enzyme sites, producing an amplification product.

In certain embodiments of the invention, the DNA polymerase is a thermophilic polymerase. In other examples of the invention, the polymerase and said nicking enzymes are stable at temperatures up to 37°C, 42°C, 60°C, 65°C, 70°C, 75°C, 80°C, or 85°C. In certain
20 embodiments, the polymerase is stable up to 60°C. The polymerase may, for example, be selected from the group consisting of Bst (large fragment), 9°N, $Vent_R^{®}$ (exo-) DNA Polymerase, Therminator, and Therminator II.

The nicking enzyme may, for example, nick upstream of the nicking enzyme binding site, or, in exemplary embodiments, the nicking enzyme may nick downstream of the nicking enzyme
25 binding site. In certain embodiments, the forward and reverse templates comprise nicking enzyme sites recognized by the same nicking enzyme and said first and said second nicking enzyme are the same. The nicking enzyme may, for example, be selected from the group consisting of Nt.BspQI, Nb.BbvCi, Nb.BsmI, Nb.BsrDI, Nb.BtsI, Nt.AlwI, Nt.BbvCI, Nt.BstNBI, Nt.CviPII, Nb.Bpu10I, and Nt.Bpu10I.

In certain aspects of the present invention, the target sequence comprises from 1 to 5 nucleotides more than the sum of the nucleotides of said forward template recognition region and said reverse template recognition region.

The DNA molecule may be, for example, genomic DNA. The DNA molecule may be, for example, selected from the group consisting of plasmid, mitochondrial, and viral DNA. In certain embodiments, the forward template is provided at the same concentration as the reverse template. In other examples, the forward template is provided at a ratio to the reverse template at the range of ratios of 1:100 to 100:1.

In other examples of the invention, the method further comprises the use of a second polymerase. The amplification may be, for example, conducted at a constant temperature. This temperature may be, for example, between 54°C and 60°C. As to the length of time for the reaction to take place, in certain examples, the amplification reaction is held at constant temperature for 1 to 10 minutes.

The present invention further comprises detecting the amplification product, for example, by a method selected from the group consisting of gel electrophoresis, mass spectrometry, SYBR I fluorescence, SYBR II fluorescence, SYBR Gold, Pico Green, TOTO-3, intercalating dye detection, FRET, molecular beacon detection, surface capture, capillary electrophoresis, incorporation of labeled nucleotides to allow detection by capture, fluorescence polarization, and lateral flow capture. The amplification products may be, for example, detected using a solid surface method, for example, where at least one capture probe is immobilized on the solid surface that binds to the amplified sequence.

The present invention may be used for multiplex amplification. Thus, for example, in certain embodiments of the present invention at least two target sequences are capable of being amplified. By "capable of being amplified" is meant the amplification reaction comprises the appropriate templates and enzymes to amplify at least two target sequences. Thus, for example, the amplification reaction may be prepared to detect at least two target sequences, but only one of the target sequences may actually be present in the sample being tested, such that both sequences are capable of being amplified, but only one sequence is. Or, where two target sequences are present, the amplification reaction may result in the amplification of both of the target sequences. The multiplex amplification reaction may result in the amplification of one,

some, or all, of the target sequences for which it comprises the appropriate templates and enzymes.

At least one of the templates, for example, may comprise a spacer, a blocking group, or a modified nucleotide.

5 Also provided as an embodiment of the present invention is a method for amplifying a single-stranded nucleic acid target sequence, comprising contacting a target nucleic acid comprising a single-stranded target sequence with a reverse template, wherein said reverse template comprises a nucleotide sequence comprising a recognition region at the 3′ end that is complementary to the 3′ end of the target sequence, a nicking enzyme site upstream of said 10 recognition region, and a stabilizing region upstream of said nicking enzyme site; providing a first nicking enzyme that is capable of nicking at the nicking enzyme site of said reverse template, and does not nick within said target sequence; providing a DNA polymerase under conditions wherein said polymerase extends said reverse template along said target sequence; contacting said extended reverse template with a forward template, wherein said forward 15 template comprises comprising a recognition region at the 3′ end that is identical to the 5′ end of the target sequence a nicking enzyme site upstream of said recognition region, and a stabilizing region upstream of said nicking enzyme site; providing a second nicking enzyme that is capable of nicking at the nicking enzyme site of said forward template and does not nick within said target sequence; under conditions wherein amplification is performed by multiple cycles of said 20 polymerase extending said forward and reverse templates along said target sequence producing a double-stranded nicking enzyme site, and said nicking enzymes nicking at said nicking enzyme sites, producing an amplification product.

Those of ordinary skill in the art understand that the examples presented herein relating to the amplification of a double-stranded nucleic acid target sequence and the detection of the 25 amplified product also apply to the amplification of a single-stranded nucleic acid target sequence and the detection of the amplified product. Further, in examples of the present invention, the target sequence may be, for example, RNA, for example, but not limited to, messenger RNA, viral RNA, microRNA, a microRNA precursor, or siRNA. In exemplary embodiments of the present invention, the polymerase comprises reverse transcription activity. 30 In yet other examples of the present invention, the target sequence is DNA, such as, for example,

genomic DNA, or for example, the target sequence is selected from the group consisting of plasmid, mitochondrial, and viral nucleic acid.

Where the method may comprise the use of more than one polymerase, in exemplary embodiments at least one of the polymerases comprises reverse transcriptase activity.

5 In other embodiments of the present invention, a set of oligonucleotide templates is provided, comprising a first template for nucleic acid amplification, comprising a recognition region at the 3′ end that is complementary to the 3′ end of a target sequence antisense strand; a nicking enzyme site upstream of said recognition region; and a stabilizing region upstream of said nicking enzyme site; and a second template for nucleic acid amplification, comprising a 10 recognition region at the 3′ end that is identical to the 5′ of said target sequence antisense strand; a nicking enzyme site upstream of said recognition region; and a stabilizing region upstream of said nicking enzyme site;

wherein said target sequence comprises from 1 to 5 spacer bases between said 3′ end of the antisense strand and said 5′ end of said antisense strand that do not bind to either template.

15 In yet other embodiments, a kit is provided for following the methods of the present invention for nucleic acid amplification, comprising a DNA polymerase; a first template for nucleic acid amplification, comprising a recognition region at the 3′ end that is complementary to the 3′ end of a target sequence antisense strand; a nicking enzyme site upstream of said recognition region; and a stabilizing region upstream of said nicking enzyme site; a second 20 template for nucleic acid amplification, comprising a recognition region at the 3′ end that is complementary to the 3′ end of a target sequence sense strand; a nicking enzyme site upstream of said recognition region; and a stabilizing region upstream of said nicking enzyme site; one or two thermostable nicking enzymes, wherein either one enzyme is capable of nicking at the nicking enzyme site of said first and said second templates, or a first enzyme is capable of 25 nicking at the nicking enzyme site of said first primer and a second enzyme is capable of nicking at the enzyme site of said second primer.

The kit may, for example, provide said polymerase, nicking enzymes, and templates in a container. The kit may provide, for example, said polymerase, nicking enzymes, and templates in two containers. In certain examples, the polymerase and nicking enzymes are in a first 30 container, and said templates are in a second container. In certain examples, the polymerase and nicking enzymes are lyophilized. The kit may, for example, further comprise instructions for

following the amplification methods of the present invention. The kit may, for example, further comprise a cuvette. The kit may, for example, further comprise a lateral flow device or dipstick. The lateral flow device or dipstick may, for example, further comprise a capture probe, wherein said capture probe binds to amplified product. The kit may, for example, further comprise a
5 detector component selected from the group consisting of a fluorescent dye, colloidal gold particles, latex particles, a molecular beacon, and polystyrene beads. In other examples, at least one of the templates of the kit comprises a spacer, blocking group, or a modified nucleotide.

Deoxynucleoside triphosphates (dNTPs) are included in the amplification reaction. One or more of the dNTPs may be modified, or labeled, as discussed herein. Nucleotides are
10 designated as follows. A ribonucleoside triphosphate is referred to as NTP or rNTP; N can be A, G, C, U or m5U to denote specific ribonucleotides. Deoxynucleoside triphosphate substrates are indicated as dNTPs, where N can be A, G, C, T, or U. Throughout the text, monomeric nucleotide subunits may be denoted as A, G, C, or T with no particular reference to DNA or RNA.

15 In another embodiment, a method is provided for nucleic acid amplification comprising forming a mixture of a target nucleic acid comprising a double-stranded target sequence having a sense strand and an antisense strand; a forward template comprising a nucleic acid sequence comprising a recognition region at the 3′ end that is complementary to the 3′ end of the target sequence antisense strand; a nicking enzyme site upstream of said recognition region, and a
20 stabilizing region upstream of said nicking enzyme site; a reverse template comprising a nucleotide sequence comprising a recognition region at the 3′ end that is complementary to the 3′ end of the target sequence sense strand, a nicking enzyme site upstream of said recognition region and a stabilizing region upstream of said nicking enzyme site; a first nicking enzyme that is capable of nicking at the nicking enzyme site of said forward template, and does not nick
25 within said target sequence; a second nicking enzyme that is capable of nicking at the nicking enzyme site of said reverse template and does not nick within said target sequence; and a thermophilic polymerase under conditions wherein amplification is performed by multiple cycles of said polymerase extending said forward and reverse templates along said target sequence producing a double-stranded nicking enzyme site, and said nicking enzymes nicking at said
30 nicking enzyme sites, producing an amplification product. In certain embodiments, the nicking

enzyme sites on the forward and reverse templates are recognized by the same nicking enzyme, and only one nicking enzyme is used for the reaction.

In another embodiment, a method is provided for nucleic acid amplification comprising forming a mixture of a target nucleic acid comprising a single-stranded target sequence; a reverse template, wherein said reverse template comprises a nucleotide sequence comprising a recognition region at the 3′ end that is complementary to the 3′ end of the target sequence, a nicking enzyme site upstream of said recognition region, and a stabilizing region upstream of said nicking enzyme site; a first nicking enzyme that is capable of nicking at the nicking enzyme site of said reverse template, and does not nick within said target sequence; a thermophilic polymerase under conditions wherein said polymerase extends said reverse template along said target sequence; a forward template, wherein said forward template comprises a nucleic acid sequence comprising a recognition region at the 3′ end that is identical to the 5′end of the target sequence; and a second nicking enzyme that is capable of nicking at the nicking enzyme site of said forward template and does not nick within said target sequence; under conditions wherein amplification is performed by multiple cycles of said polymerase extending said forward and reverse templates along said target sequence producing a double-stranded nicking enzyme site, and said nicking enzymes nicking at said nicking enzyme sites, producing an amplification product. In certain embodiments, the nicking enzyme sites on the forward and reverse templates are recognized by the same nicking enzyme, and only one nicking enzyme is used for the reaction.

In other embodiments of the invention are provided methods for the separation of amplified nucleic acids obtained by the amplification methods of the invention. In yet further embodiments of the invention are provided methods for detecting and/or analyzing the amplified nucleic acids obtained by the amplification methods of the invention, including, for example, methods using SYBR I, II, SYBR Gold, Pico Green, TOTO-3, and most intercalating dyes, molecular beacons, FRET, surface capture using immobilized probes with fluorescence, electrochemical, or colorimetric detection, mass spectrometry, capillary electrophoresis, the incorporation of labeled nucleotides to allow detection by capture or fluorescence polarization, lateral flow, and other methods involving capture probes. Methods using capture probes for detection include, for example, the use of a nucleic acid molecule (the capture probe) comprising a sequence that is complementary to the amplified product such that the capture probe binds to

amplified nucleic acid. The reaction may, for example, further comprise an antibody directed against a molecule incorporated into or attached to the capture probe. Or, for example, the capture probe, or a molecule that binds to the capture probe, may incorporate, for example, an enzyme label, for example, peroxidase, alkaline phosphatase, or beta-galactosidase, a fluorescent
5 label, such as, for example, fluorescein or rhodamine, or, for example, other molecules having chemiluminescent or bioluminescent activity. The embodiments of the present invention also comprise combinations of these detection and analysis methods.

Brief Description of the Drawings
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Figures 1A-D are graphic drawings depicting mechanisms of the reactions of the present invention. Figure 1D is a legend for Figure 1.

Figure 2. 20% polyacrylamide gel of reaction products from a DNA NEAR assay.

The NEAR reaction was run for 2.5 minutes at 56 °C, then heat denatured at 94 °C for 4
15 minutes. Six μL of the reaction was run on a 20% polyacrylamide gel at 160V for ~2.5 hrs. The gel was stained with SYBR II gel stain. Lane 1: NEAR reaction no target control for 25mer assay. Lane 2: NEAR reaction no target control for 27mer assay. Lane 3: NEAR reaction for 25mer assay with 3.5E+5 copies of genomic *Bacillus subtilis* DNA. Lane 4: NEAR reaction for 27mer assay with 1.1E+6 copies of genomic *Bacillus subtilis* DNA.

20 **Figure 3.** 20% polyacrylamide gel of reaction products from an RNA NEAR assay.

The NEAR reaction was run for 12 minutes at 56 °C, then heat denatured at 94 °C for 4 minutes. Six μL of the reaction was run on a 20% polyacrylamide gel at 160V for ~2.5 hrs. The gel was stained with SYBR II gel stain. Lane 1 & 2: NEAR reaction for 25mer assay with 1E+6 copies of Ebola Armored RNA (Ambion). Lane 3 & 4: NEAR reaction no target control for
25 25mer assay. 25mer reaction products are outlined in the white box.

Figure 4. Mass Spectrum of *Bacillus anthracis* DNA assay products.

A) 0 copies of target or B) 5E+5 copies of genomic DNA added to the NEAR reaction. The NEAR reaction was run for 10 minutes, then heat denatured at 94 °C for 4 minutes. Ten micro liters of sample was injected into the LC/ESI-MS. The (-4) charge state of the 26mer
30 product and its complementary sequence are outlined in a black box. The smaller adjacent peaks are the sodium adducts of the main product.

Figure 5. Mass Spectrum of MS2 genomic RNA assay products.

A) 0 copies of target, B) 1E+6 copies of MS2 genomic RNA, or C) 1E+6 copies of synthetic target DNA added to the NEAR reaction. The NEAR reaction was run for 10 minutes, then heat denatured at 94 °C for 4 minutes. Ten micro liters of sample was injected into the LC/ESI-MS. The (-4) charge state of the 27mer product and its complement sequence are outlined in a black box. The smaller adjacent peaks are the sodium adducts of the main product.

Figure 6. Real-time detection of NEAR assay amplification using intercalating fluorescent dyes.

Real-time amplification of *Yersinia pestis* genomic DNA at 500 copies (squares) compared to the no target control (NTC, open triangles). The reaction was run for 10 minutes at 58 °C and monitored by the real-time fluorescence with SYBR II (n = 5).

Figure 7. Real-time detection of NEAR assay amplification using fluorescence resonance energy transfer (FRET).

Real-time amplification of *Yersinia pestis* synthetic DNA at 10,000 copies (squares) compared to the no target control (NTC, open triangles). The reaction was run for 10 minutes at 57 °C, n = 3.

Figure 8. Francisella *tularensis* assay amplification detected in real-time using molecular beacons.

Either 0 copies (open triangles) or 1E+5 copies (squares) were added to the reaction mix and run for 10 minutes at 57.5 °C.

Figure 9. False alarm rate testing results comparing average AUC values.

Error bars denote one standard deviation. *Bacillus subtilis* NEAR assays were run for 10 min at 55 °C in the presence and absence of *Bacillus subtilis* genomic DNA. Enzymes were heat denatured at 94 °C for 4 min. A 10 μL sample was injected into the LC/ESI-MS and the area under the curve (AUC) of the product peaks were analyzed. True Positives contained 10,000 copies of *Bacillus subtilis* along with 990,000 copies of near neighbor (*Bacillus thuringiensis*). True Negatives contained 10,000 copies of *E. coli* with 990,000 copies of near neighbor, and water negatives contained no DNA as a control.

Figure 10. Replication study of the NEAR Assay using molecular beacon detection with different operators performing the experiments on two different days.

The NEAR reaction was run for 10 minutes at 57.5 °C (in the presence and absence of 500 copies of *Francisella tularensis* genomic DNA) with a 4 min heat kill at 94 °C. 300nM molecular beacon was added and monitored at 45, 50, and 57 °C (n = 24).

Figure 11. Sensitivity of the NEAR reaction using molecular beacon detection.

5 The NEAR assay was run for 10 minutes 57.5 °C. The reaction was stopped with a 4 min heat denaturation step at 94 °C. 300nM molecular beacon was added and the fluorescence was monitored at 57.5 °C (n = 3). Fluorescence was monitored for beacon opening in the presence NEAR reactions amplified with 1E+6, 5E+5, 5E+4, 5E+2, 50, and 0 (NTC) input copies of *Francisella tularensis* genomic DNA, and compared to the background fluorescence of the 10 beacon alone (MB).

Figure 12. Final concentration of amplified products in the NEAR reaction.

The NEAR reaction was run for 10 min at 55 °C with varying copies of *Bacillus subtilis* genomic DNA. The reaction was stopped with a heat denaturation step at 94 °C for 4 minutes. A 10 μL sample was injected into the LC/ESI-MS and the AUC of the product peak at 1944 15 Daltons was analyzed and compared to a standard curve.

Figure 13. Correlation of the input RNA target copy number to the final concentration of amplified products.

The Ebola NEAR assay was run for 12 min at 55 °C with varying copies of synthetic RNA corresponding to the Ebola genome DNA. The reaction was stopped with a heat 20 denaturation step at 94 °C for 4 minutes. A 10 μL sample was injected into the LC/ESI-MS and the AUC of the product peak at 1936 Daltons was analyzed and compared to the standard curve of AUC values. (n = 3)

Figure 14. Mass spec product analysis demonstrating NEAR reaction specificity.

The *Bacillus anthracis* NEAR reaction was run in the presence of a dilution of copies of 25 Bacillus thuringiensis for 10 min at 56 °C (n = 3), then heat denatured at 94 °C for 4 minutes. A 10 μL sample was injected into the LC/ESI-MS and AUC values of product peaks analyzed.

Figure 15. The effect of an interferent panel on the NEAR amplification.

Bacillus subtilis NEAR DNA reactions were run for 10 min at 55 °C and heated to 94 °C for 4 minutes to stop the reaction. Reactions were run in triplicate in the presence 1E+5 copies of 30 *Bacillus subtilis* genomic DNA ("_1E+5") or with no target DNA present ("_0"). Sample x is

the control assay with no interferent added. Interferents A through F were added at 50% reaction volume to the *Bacillus subtilis* assay. The AUC of mass spec product peaks were analyzed using a two-way ANOVA and Bonferroni t-test. (Key: A = none; B = House dust, skim milk; C = AZ test dust, humic acid; D = Diesel soot; E = Skim milk; F = Mold spores)

5 **Figure 16.** Gel electrophoresis results for the *Bacillus subtilis / Bacillus anthracis* DNA duplex reaction.

The NEAR reaction including templates for both *Bacillus subtilis (Bs)* and *Bacillus anthracis (Ba)* assays was run in the absence of target DNA (negative), in the presence of *Bacillus subtilis* only (positive for 27mer product), and in the presence of both *Bacillus subtilis*
10 and *Bacillus anthracis* (positive for 27mer and 25mer product respectively). The target copy number used in this assay was 500,000 copies. The assay was run for 10 min at 57 °C. Templates varied in concentration between the assays to control the amplification (100nM for *Bacillus anthracis* and 50 nM for *Bacillus subtilis*). Samples were run on a 20% polyacrylamide gel at 160 V for ~2 hours. The gel was stained with SYBR II fluorescent dye and imaged. The
15 fluorescent bands were quantitated and analyzed as the integrated optical density (IOD) (n = 8).

Figure 17. Specificity results for the *Bacillus subtilis / Bacillus anthracis* DNA duplex reaction shown by gel electrophoresis.

The NEAR reaction including templates for both a *Bacillus subtilis (Bs)* and *Bacillus anthracis (Ba)* assay was run in the absence of target DNA (negative), in the presence of
20 *Bacillus subtilis* only (27mer product), and in the presence of both *Bacillus subtilis* and *Bacillus anthracis* (27mer and 25mer product respectively). The target copy number for each genome present in this assay was 500,000 copies. All reactions contained 500,000 copies of *Bacillus thuringiensis* as clutter. Templates varied in concentration between the assays to control the amplification. The assay was run for 10 min at 57 °C, heat denatured at 94 °C for 4 min, and 6
25 μL was loaded on to a 20% gel run at 160 V for ~2 hours. The gel was stained with SYBR II fluorescent dye and imaged. The fluorescent bands were quantitated and analyzed as the integrated optical density (IOD).

Figure 18. Gel electrophoresis results for the MS2/Ebola RNA duplex reaction.

The NEAR reaction including templates for both a MS2 and Ebola assay was run in the
30 absence of target RNA (negative, lanes 2-5), in the presence of MS2 only (27mer product, lanes 6 and 7), and in the presence of both MS2 and Ebola RNA (27mer and 25mer product

respectively, lanes 8 and 9). The target copy number used in this assay was 1E+6 copies. The assay was run for 10 min at 57 °C. Templates varied in concentration between the assays to control the amplification. Samples were run on a 20% polyacrylamide gel at 160 V for ~2.5 hours. The gel was stained with SYBR II fluorescent dye and imaged. The fluorescent bands were quantitated and analyzed as the integrated optical density (IOD).

Figure 19. Mass spec analysis of NEAR amplification of DNA from lysed spores.

Average AUC values from amplified product masses compared for lysed and unlysed samples. Lysed spore samples were then added to NEAR master mix and run for 10 minutes at 55 °C, heat denatured for 4 minutes at 94 °C, and run on the mass spec for analysis. AUC values of product peaks were averaged and compared (n = 3).

Figure 20. Demonstration of the capture and extension approach for surface detection of the NEAR assay.

A.) Average binding (NEAR positive reaction product with no added polymerase), B.) 500,000 target (NEAR positive reaction product with added polymerase), and C.) No target (NEAR negative reaction with added polymerase) are compared. The NEAR assay was run for 10 minutes at 55 °C, heat denatured at 94 °C for 4 minutes, then added to the plate with capture probe bound to the surface on the 5′ end. Polymerase is added to one well of the positive reaction. The plate is incubated at 55 °C for 30 min, washed, SYBR II added, washed 3 times, and read on a Tecan plate reader (495 nm excitation/530 nm emission).

Figure 21. Pseudo-real-time fluorescence detection of the NEAR FRET assay with a single template immobilized on a surface in the presence (squares) and absence (open triangles) of 1E+6 copies of genomic DNA.

NEAR reaction was performed in flat bottom 96-well plates covered with neutravidin. Solution of 1 μM FRET-labeled reverse template was incubated with gentle mixing for 1 hr at 37 °C. Wells were washed 3 times with a PBS-Tween solution to release unbound template. NEAR reaction mix was added to the wells (one for each time point taken) and incubated at 58 °C on a heating block in a shaking incubator set to 135 RPM. Time points were taken by adding 1 μL EDTA to the well to stop the reaction. The fluorescence was read from the bottom using a Tecan 100 plate reader.

Detailed Description

Provided herein are methods for the exponential amplification of short DNA or RNA sequences.

Target nucleic acids of the present invention include double-stranded and single-stranded nucleic acid molecules. The nucleic acid may be, for example, DNA or RNA. Where the target nucleic acid is an RNA molecule, the molecule may be, for example, double-stranded, single-stranded, or the RNA molecule may comprise a target sequence that is single-stranded. Target nucleic acids include, for example, genomic, plasmid, mitochondrial, cellular, and viral nucleic acid. The target nucleic acid may be, for example, genomic, chromosomal, plasmid DNA, a gene, any type of cellular RNA, or a synthetic oligonucleotide. By "genomic nucleic acid" is meant any nucleic acid from any genome, for example, including animal, plant, insect, and bacterial genomes, including, for example, genomes present in spores. Target nucleic acids further include microRNAs and siRNAs.

MicroRNAs, miRNAs, or small temporal RNAs (stRNAs), are short single-stranded RNA sequences, about 21-23 nucleotides long that are involved in gene regulation. MicroRNAs are thought to interfere with the translation of messenger RNAs as they are partially complementary to messenger RNAs. (see, for example, Ruvkun, Gl, Science 294:797-99 (2001); Lagos-Quintana, M., et al., Science 294:854-58 (2001); Lau, N.C., et al, Science 294:858-62 (2001); Lee, R.C., and Ambros, V., Science 294:862-64 (2001); Baulcombe, D., et al., Science 297:2002-03 (2002); Llave, C., Science 297:2053-56 (2002); Hutvagner, G., and Zamore, P.D., Science 297:2056-60 (2002)). MicroRNA may also have a role in the immune system, based on studies recently reported in knock-out mice. (see, for example, Wade, N., "Studies Reveal and Immune System Regulator" New York Times, April 27, 2007). MicroRNA precursors that may also be detected using the methods of the present invention include, for example, the primary transcript (pri-miRNA) and the pre-miRNA stem-loop-structured RNA that is further processed into miRNA.

Short interfering RNAs, or siRNAs are at least partially double-stranded, about 20-25 nucleotide long RNA molecules that are found to be involved in RNA interference, for example, in the down-regulation of viral replication or gene expression (see for example Zamore et al., 2000, Cell, 101, 25-33; Bass, 2001, Nature, 411, 428-429; Elbashir et al., 2001, Nature, 411, 494-498; and Kreutzer et al., International PCT Publication No. WO 00/44895; Zernicka-Goetz

et al., International PCT Publication No. WO 01/36646; Fire, International PCT Publication No. WO 99/32619; Plaetinck et al., International PCT Publication No. WO 00/01846; Mello and Fire, International PCT Publication No. WO 01/29058; Deschamps-Depaillette, International PCT Publication No. WO 99/07409; and Li et al., International PCT Publication No. WO 00/44914; Allshire, 2002, Science, 297, 1818-1819; Volpe et al., 2002, Science, 297, 1833-1837; Jenuwein, 2002, Science, 297, 2215-2218; and Hall et al., 2002, Science, 297, 2232-2237; Hutvagner and Zamore, 2002, Science, 297, 2056-60; McManus et al., 2002, RNA, 8, 842-850; Reinhart et al., 2002, Gene & Dev., 16, 1616-1626; and Reinhart & Bartel, 2002, Science, 297, 1831).

The use of the term "target sequence" may refer to either the sense or antisense strand of the sequence, and also refers to the sequences as they exist on target nucleic acids, amplified copies, or amplification products, of the original target sequence. The amplification product may be a larger molecule that comprises the target sequence, as well as at least one other sequence, or other nucleotides. The length of the target sequence, and the guanosine:cytosine (GC) concentration (percent), is dependent on the temperature at which the reaction is run; this temperature is dependent on the stability of the polymerases and nicking enzymes used in the reaction. Those of ordinary skill in the art may run sample assays to determine the appropriate length and GC concentration for the reaction conditions. For example, where the polymerase and nicking enzyme are stable up to 60ºC, then the target sequence may be, for example, from 19 to 50 nucleotides in length, or for example, from 20 to 45, 20 to 40, 22-35, or 23 to 32 nucleotides in length. The GC concentration under these conditions may be, for example, less than 60%, less than 55%, less than 50%, or less than 45%. The target sequence should not contain nicking sites for any nicking enzymes that will be included in the reaction mix.

The target sequences may be amplified in many types of samples including, but not limited to samples containing spores, viruses, cells, nucleic acid from prokaryotes or eukaryotes, or any free nucleic acid. For example, the assay can detect the DNA on the outside of spores without the need for lysis. The sample may be isolated from any material suspected of containing the target sequence. For example, for animals, for example, mammals, such as, for example, humans, the sample may comprise blood, bone marrow, mucus, lymph, hard tissues, for example, liver, spleen, kidney, lung, or ovary, biopsies, sputum, saliva, tears, feces, or urine.

Or, the target sequence may be present in air, plant, soil, or other materials suspected of containing biological organisms.

Target sequences may be present in samples that may also contain environmental and contaminants such as dust, pollen, and diesel exhaust, or clinically relevant matrices such as urine, mucus, or saliva. Target sequences may also be present in waste water, drinking water, air, milk, or other food. Depending on the concentration of these contaminants, sample purification methods known to those of ordinary skill in the art may be required to remove inhibitors for successful amplification. Purification may, for example, involve the use of detergent lysates, sonication, vortexing with glass beads, or a French press. This purification could also result in concentration of the sample target. Samples may also, for be further purified, for example, by filtration, phenol extraction, chromatography, ion exchange, gel electrophoresis, or density dependent centrifugation. The sample can be added directly to the reaction mix or pre-diluted and then added.

An oligonucleotide is a molecule comprising two or more deoxyribonucleotides or ribonucleotides, for example, more than three. The length of an oligonucleotide will depend on how it is to be used. The oligonucleotide may be derived synthetically or by cloning.

The term “complementary” as it refers to two nucleic acid sequences generally refers to the ability of the two sequences to form sufficient hydrogen bonding between the two nucleic acids to stabilize a double-stranded nucleotide sequence formed by hybridization of the two nucleic acids.

As used herein, "hybridization" and "binding" are used interchangeably and refer to the non-covalent binding or "base pairing" of complementary nucleic acid sequences to one another. Whether or not a particular probe remains base paired with a polynucleotide sequence depends on the degree of complementarity, the length of the probe, and the stringency of the binding conditions. The higher the stringency, the higher must be the degree of complementarity, and/or the longer the probe for binding or base pairing to remain stable.

As used herein, "stringency" refers to the combination of conditions to which nucleic acids are subjected that cause double-stranded nucleic acid to dissociate into component single strands such as pH extremes, high temperature, and salt concentration. The phrase "high stringency" refers to hybridization conditions that are sufficiently stringent or restrictive such that only specific base pairings will occur. The specificity should be sufficient to allow for the

detection of unique sequences using an oligonucleotide probe or closely related sequence under standard Southern hybridization protocols (as described in J. Mol. Biol. 98:503 (1975)).

Templates are defined as oligonucleotides that bind to a recognition region of the target and also contain a nicking enzyme binding region upstream of the recognition region and a stabilizing region upstream to the nicking enzyme binding region.

By "recognition region" is meant a nucleic acid sequence on the template that is complementary to a nucleic acid sequence on the target sequence. By "recognition region on the target sequence" is meant the nucleotide sequence on the target sequence that is complementary to, and binds to, the template.

By "stabilizing region" is meant a nucleic acid sequence having, for example, about 50% GC content, designed to stabilize the molecule for, for example, the nicking and/or extension reactions.

In describing the positioning of certain sequences on nucleic acid molecules, such as, for example, in the target sequence, or the template, it is understood by those of ordinary skill in the art that the terms "3′ " and "5′ " refer to a location of a particular sequence or region in relation to another. Thus, when a sequence or a region is 3′ to or 3′ of another sequence or region, the location is between that sequence or region and the 3′ hydroxyl of that strand of nucleic acid. When a location in a nucleic acid is 5′ to or 5′ of another sequence or region, that means that the location is between that sequence or region and the 5′ phosphate of that strand of nucleic acid.

The polymerase is a protein able to catalyze the specific incorporation of nucleotides to extend a 3′ hydroxyl terminus of a primer molecule, such as, for example, the template oligonucleotide, against a nucleic acid target sequence. The polymerase may be, for example, thermophilic so that it is active at an elevated reaction temperature. It may also, for example, have strand displacement capabilities. It does not, however, need to be very processive (30-40 nucleotides for a single synthesis is sufficient). If the polymerase also has reverse transcription capabilities (such as Bst (large fragment), 9°N, Therminator, Therminator II, etc.) the reaction can also amplify RNA targets in a single step without the use of a separate reverse transcriptase. More than one polymerase may be included in the reaction, in one example one of the polymerases may have reverse transcriptase activity and the other polymerase may lack reverse transcriptase activity. The polymerase may be selected from, for example, the group consisting of one or more of the polymerases listed in Table 1.

Table 1

Polymerase
Bst DNA polymerase
Bst DNA polymerase (Large fragment)
9°Nm DNA polymerase
Phi29 DNA polymerase
DNA polymerase I (*E.coli*)
DNA polymerase I, Large (Klenow) fragment
Klenow fragment (3′-5′ exo-)
T4 DNA polymerase
T7 DNA polymerase
Deep $Vent_R$™ (exo-) DNA Polymerase
Deep $Vent_R$™ DNA Polymerase
DyNAzyme™ EXT DNA
DyNAzyme™ II Hot Start DNA Polymerase
Phusion™ High-Fidelity DNA Polymerase
Therminator™ DNA Polymerase
Therminator™ II DNA Polymerase
$Vent_R^{®}$ DNA Polymerase
$Vent_R^{®}$ (exo-) DNA Polymerase
RepliPHI™ Phi29 DNA Polymerase

rBst DNA Polymerase
rBst DNA Polymerase, Large Fragment (IsoTherm™ DNA Polymerase)
MasterAmp™ AmpliTherm™ DNA Polymerase
Taq DNA polymerase
Tth DNA polymerase
Tfl DNA polymerase
Tgo DNA polymerase
SP6 DNA polymerase
Tbr DNA polymerase
DNA polymerase Beta
ThermoPhi DNA polymerase

"Nicking" refers to the cleavage of only one strand of the double-stranded portion of a fully or partially double-stranded nucleic acid. The position where the nucleic acid is nicked is referred to as the nicking site or nicking enzyme site. The recognition sequence that the nicking 5 enzyme recognizes is referred to as the nicking enzyme binding site. "Capable of nicking" refers to an enzymatic capability of a nicking enzyme.

The nicking enzyme is a protein that binds to double-stranded DNA and cleaves one strand of a double-stranded duplex. The nicking enzyme may cleave either upstream or downstream of the binding site, or nicking enzyme recognition site. In exemplary embodiments, 10 the reaction comprises the use of nicking enzymes that cleave or nick downstream of the binding site (top strand nicking enzymes) so that the product sequence does not contain the nicking site. Using an enzyme that cleaves downstream of the binding site allows the polymerase to more easily extend without having to displace the nicking enzyme. The nicking enzyme must be functional in the same reaction conditions as the polymerase, so optimization between the two 15 ideal conditions for both is necessary. Nicking enzymes are available from, for example, New England Biolabs (NEB) and Fermentas. The nicking enzyme may, for example, be selected from the group consisting of one or more of the nicking enzymes listed in Table 2.

Table 2

Nicking Enzyme	Alternate Name
Nb.BbvCI	
Nb.Bpu10I	
Nb.BsaI	
Nb.BsmI	
Nb.BsrDI	
Nb.BstNBIP	
Nb.BstSEIP	
Nb.BtsI	
Nb.SapI	
Nt.AlwI	
Nt.BbvCI	
Nt.BhaIIIP	
Nt.Bpu10I	
Nt.Bpu10IB	
Nt.BsaI	
Nt.BsmAI	
Nt.BsmBI	
Nt.BspD6I	
Nt.BspQI	
Nt.Bst9I	
Nt.BstNBI	N.BstNB I
Nt.BstSEI	
Nt.CviARORFMP	
Nt.CviFRORFAP	
Nt.CviPII	Nt.CviPIIm
Nt.CviQII	
Nt.CviQXI	
Nt.EsaSS1198P	
Nt.MlyI	
Nt.SapI	

Nicking enzymes may be, for example, selected from the group consisting of Nt.BspQI(NEB), Nb.BbvCI(NEB), Nb.BsmI(NEB), Nb.BsrDI(NEB), Nb.BtsI(NEB),
5 Nt.AlwI(NEB), Nt.BbvCI(NEB), Nt.BstNBI(NEB), Nt.CviPII(NEB), Nb.Bpu101(Fermantas), and Nt.Bpu101(Fermentas). In certain embodiments, the nicking enzyme is selected from the group consisting of Nt.NBst.NBI, Nb.BsmI, and Nb.BsrDI. Those of ordinary skill in the art are aware that various nicking enzymes other than those mentioned specifically herein may be used in the methods of the present invention.

Nicking enzymes and polymerases of the present invention may be, for example, stable at room temperature, the enzymes may also, for example, be stable at temperatures up to 37ºC , 42ºC , 60ºC, 65ºC, 70ºC, 75ºC, 80ºC, or 85ºC. In certain embodiments, the enzymes are stable up to 60ºC.

5 Product or amplified product is defined as the end result of the extension of the template along the target that is nicked, released, and then feeds back into the amplification cycle as a target for the opposite template.

A “native nucleotide” refers to adenylic acid, guanylic acid, cytidylic acid, thymidylic acid, or uridylic acid. A “derivatized nucleotide” is a nucleotide other than a native nucleotide.

10 The reaction may be conducted in the presence of native nucleotides, such as, for example, dideoxyribonucleoside triphosphates (dNTPs). The reaction may also be carried out in the presence of labeled dNTPs, such as, for example, radiolabels such as, for example, ^{32}P, ^{33}P, ^{125}I, or ^{35}S, enzyme labels such as alkaline phosphatase, fluorescent labels such as fluorescein isothiocyanate (FITC), biotin, avidin, digoxigenin, antigens, haptens, or fluorochromes. These 15 derivatized nucleotides may, for example, be present in the templates.

By “constant temperature,” “isothermal conditions” or “isothermally” is meant a set of reaction conditions where the temperature of the reaction is kept essentially constant during the course of the amplification reaction. An advantage of the amplification method of the present invention is that the temperature does not need to be cycled between an upper temperature and a 20 lower temperature. The nicking and the extension reaction will work at the same temperature or within the same narrow temperature range. However, it is not necessary that the temperature be maintained at precisely one temperature. If the equipment used to maintain an elevated temperature allows the temperature of the reaction mixture to vary by a few degrees, this is not detrimental to the amplification reaction, and may still be considered to be an isothermal 25 reaction.

The term “multiplex amplification” refers to the amplification of more than one nucleic acid of interest. For example, it can refer to the amplification of multiple sequences from the same sample or the amplification of one of several sequences in a sample as discussed, for example, in U.S. Patent Nos. 5,422,252; and 5,470,723, which provide examples of multiplex 30 strand displacement amplification. The term also refers to the amplification of one or more sequences present in multiple samples either simultaneously or in step-wise fashion.

Template Design

Forward and Reverse templates are designed so that there is a stabilizing region at the 5′ end, a nicking site downstream of the stabilizing region, and a recognition region downstream of the nicking site on the 3′ end of the oligonucleotide. The total oligo length can range from 19 to 40, for example from 19-40, 23-40, 20-24, 23-24, 23-32, 25-40, 27-40, or 27-35 nucleotides depending on the length of each individual region, the temperature, the length of the target sequence, and the GC concentration. The templates may be designed so that they, together, would bind to less than or equal to 100% of the target sequence, one binding to the sense strand, and one to the antisense strand. For example, where the forward template binds to about 60% of the target antisense strand, the reverse template may, for example, bind to about 40% of the target sense strand. The templates may be designed to allow for spacer bases on the target sequence, that do not bind to either template. The templates thus may be designed to bind to about 30%, about 40%, about 50%, or about 60% of the target sequence.

The recognition region of the forward template is designed to be identical to the 5′ region of the target sense strand and complementary to the 3′ end of the target site antisense strand, for example, 8-16, 9-16, 10-16, 10-15, or 11-14 nucleotides long. In exemplary embodiments, the length is 12-13 nucleotides. The recognition region of the reverse template is designed to be complementary to the 3′ end of the target site sense strand, for example, 8-16, 9-16, 10-16, 10-15, or 11-14 nucleotides long. In exemplary embodiments, the length is 12-13 nucleotides.

In certain embodiments, the lengths of the recognition regions are adjusted so that there is at least one nucleotide in the target sequence that is not in the forward template's recognition region and also does not have its complement in the reverse template's recognition region. These spacer bases are nucleotides contained within the target sequence that lie in between the 3′ ends of the forward and reverse templates. In certain embodiments, 5 spacer bases or less are present in the target sequence. In exemplary embodiments, the number of spacer bases is 2 to 3. In certain embodiments, the number of spacer bases is 1, 2, 3, 4, or 5. These spacer bases allow for distinction of the true amplified product from any background products amplified by extension due to overlapping templates in a similar manner to primer-dimers. This consideration allows for improved discrimination between background and amplification of true target. However, these spacer bases are not required for the amplification to proceed.

The nicking site sequence of the template depends on which nicking enzyme is chosen for each template. Different nicking enzymes may be used in a single assay, but a simple amplification may, for example, employ a single nicking enzyme for use with both templates. Thus, the embodiments of the present invention include those where both templates comprise recognition sites for the same nicking enzyme, and only one nicking enzyme is used in the reaction. In these embodiments, both the first and second nicking enzymes are the same. The present invention also includes those embodiments where each template comprises a recognition site for a different nicking enzyme, and two nicking enzymes are used in the reaction.

For example, in the case of Nt.BstNBI, the enzyme binding site is 5′-GAGTC-3′ and the enzyme nicks the top strand four nucleotides down stream of this site (*i.e.,* GAGTCNNNN^). The amplification reaction shows little dependence on the sequence of these four nucleotides (N), though optimal sequence of this region is 25% or less GC content and with a thymine adjacent to the 5′ nucleotide of the binding region. The latter stipulation allows for the priming ability of products that have an additional adenine added on by the polymerase. The sequence of the four nucleotides can be optimized to create or eliminate the presence of hairpins, self-dimers, or heterodimers, depending on the application.

The stabilizing region on the 5′ end of the template oligonucleotide is designed to be roughly 50% GC. Thus, the GC content may be, for example, about 40%-60%, about 42%-58%, about 44%-56%, about 46%-54%, about 48%-52%, or about 49%-51%. These parameters result in a stabilizing region length of 8-11 nucleotides for the Nt.BstNBI enzyme, though lengths as short as 6 and as long as 15 nucleotides have been tested and were shown to work in this amplification method. Longer stabilizing regions or increased %GC to greater than 50 % could further stabilize the nicking and extension reactions at higher reaction temperatures. The sequence of the 5′ stabilizing regions of forward and reverse templates are usually identical, but can be varied if the aim is to capture each product strand independently. The sequence of this region should not interfere with the nicking site or the recognition region, though short internal hairpins within the template sequence have been shown to have improved real-time results.

The templates of the present invention may include, for example, spacers, blocking groups, and modified nucleotides. Modified nucleotides are nucleotides or nucleotide triphosphates that differ in composition and/or structure from natural nucleotide and nucleotide triphosphates. Modified nucleotide or nucleotide triphosphates used herein may, for example, be

modified in such a way that, when the modifications are present on one strand of a double-stranded nucleic acid where there is a restriction endonuclease recognition site, the modified nucleotide or nucleotide triphosphates protect the modified strand against cleavage by restriction enzymes. Thus, the presence of the modified nucleotides or nucleotide triphosphates encourages
5 the nicking rather than the cleavage of the double-stranded nucleic acid. Blocking groups are chemical moieties that can be added to the template to inhibit target sequence-independent nucleic acid polymerization by the polymerase. Blocking groups are usually located at the 3′ end of the template. Examples of blocking groups include, for example, alkyl groups, non-nucleotide linkers, phosphorothioate, alkane-diol residues, peptide nucleic acid, and nucleotide derivatives
10 lacking a 3′-OH, including, for example, cordycepin. Examples of spacers, include, for example, C3 spacers. Spacers may be used, for example, within the template, and also, for example, at the 5′ end, to attach other groups, such as, for example, labels.

Detailed Mechanism of Amplification

15 NEAR amplification requires the presence of a nucleic acid target, at least two template oligonucleotides, a thermophilic nicking enzyme, a thermophilic polymerase, and buffer components all held at the reaction temperature. The recognition region of the templates interacts with the complementary target sequence. Since the melting temperature of the complementary regions of the target and template is well below the reaction temperature, the
20 interaction between the two nucleic acid strands is transient, but allows enough time for a thermophilic polymerase to extend from the 3′ end of the template along the target strand. Experiments have shown that certain polymerases bind to single-stranded oligonucleotides. The pre-formation of this complex can facilitate the speed of the amplification process.

For a double-stranded target, both templates can interact with the corresponding target
25 strands simultaneously (forward template with the antisense strand and reverse template with the sense strand) during the normal breathing of double-stranded DNA. The target may also be generated by a single or double nick sites within the genome sequence. For a single-stranded target (either RNA or DNA), the reverse template binds and extends first (Figure 1, Step 1 and 2). The extended sequence contains the complement to the forward template. The forward
30 template then displaces a region of the target and binds to the 3′ synthesized region complementary to the recognition region of the forward template (Step 3). Alternatively, another

reverse template can also displace the initial extended reverse template at the recognition region to create a single-stranded extended reverse template for the forward template to bind. The initial binding and extension of the templates is facilitated by a non-processive polymerase that extends shorter strands of DNA so that the melting temperature of the synthesized product is at or near the reaction temperature; therefore, a percentage of the product becomes single-stranded once the polymerase dissociates. The single-stranded product is then available for the next template recognition site to bind and polymerase to extend.

The forward template is extended to the 5′ end of the reverse template, creating a double-stranded nicking enzyme binding site for the reverse template (Step 5). The nicking enzyme then binds to the duplex and nicks directly upstream of the recognition sequence of the reverse template strand (in the case of a top-strand nicking enzyme) (Step 6). The nucleic acid sequence downstream of the nick is either released (if the melting temperature is near the reaction temperature) and/or is displaced by the polymerase synthesis from the 3′-OH nick site. Polymerase extends along the forward template to the 5′ end of the forward template (Step 8). The double-strand formed from the extension of both templates creates a nicking enzyme binding site on either end of the duplex. This double-strand is termed the NEAR amplification duplex. When nicking enzyme binds and nicks, either the target product located in between the two nick sites (with 5′-phosphate and 3′-OH) is released, usually ranging in length from (but is not limited to) 23 to 29 bases (Steps 9-11A), or the singly-nicked product containing the target product and the reverse complement of the nick site and stability region of the template (usually 36 to 48 bases in length) is released (Steps 9-11B). The ratio of products 1 to 2 can be adjusted by varying the concentrations of the templates. The forward:reverse template ratio may vary from, for example, molar ratios of 100:1, 75:1; 50:1, 40:1, 30:1, 20:1, 10:1, 5:1, 2.5:1, 1:1, 1:2.5, 1:5, 1:10, 1:20, 1:30, 1:40, 1:50, 1:75, or 1:100. The ratio of products (A to B) is dependent on the ratio of nicking enzyme to polymerase, *i.e.* a higher concentration of polymerase results in more of the longer length product (B) since there is comparatively less nicking enzyme to nick both strands simultaneously before the polymerase extends. Since displaced/released product of the reverse template feeds into the forward template and vice versa, exponential amplification is achieved. The nicking enzyme:polymerase ratio may vary from, for example, enzyme unit ratios of 20:1, 15:1; 10:1, 5:1, 4:1, 3:1, 2:1, 1.5:1, 1:1, 1:1.5, 1:2, 1:3, 1:4, 1:5, 1:10, 1:15, 1:20. In certain embodiments, the ratio of nicking enzyme to polymerase may, for example, be 1:3,

1:2, 1:1.5, or 1:0.8. Those of ordinary skill in the art recognize that these ratios may represent rounded values. This nicking and polymerase extension process continues until one of the resources (usually dNTPs or enzyme) is exhausted.

The time that the reaction is run may vary from, for example, 1-20 minutes, or 1-10, 1-8, 1-5, 1-2.5, 2.5-5, 2.5-8, 2.5-10, or 2.5-20 minutes.

The methods of the present invention do not require the use of temperature cycling, as often is required in methods of amplification to dissociate the target sequence from the amplified nucleic acid. The temperature of the reaction may vary based on the length of the sequence, and the GC concentration, but, as understood by those of ordinary skill in the art, the temperature should be high enough to minimize non-specific binding. The temperature should also be suitable for the enzymes of the reaction, the nicking enzyme and the polymerase. For example, the reaction may be run at 37°C-85°C, 37°C -60°C, 54°C -60°C, and, in exemplary embodiments, from 55°C -59°C.

The polymerase may be mixed with the target nucleic acid molecule before, after, or at the same time as, the nicking enzyme. In exemplary embodiments, a reaction buffer is optimized to be suitable for both the nicking enzyme and the polymerase.

Reactions may be allowed to completion, that is, when one of the resources is exhausted. Or, the reaction may be stopped using methods known to those of ordinary skill in the art, such as, for example, heat denaturation, or the addition of EDTA, high salts, or detergents. In exemplary embodiments, where mass spectrometry is to be used following amplification, EDTA may be used to stop the reaction.

Reaction Components

In a 1.5 mL Eppendorf tube combine the following reagents in order from top to bottom:

Reagent Added:	µL Per Reaction
H_2O	31.4
10X Thermopol Buffer (NEB)	5
10X NEB Buffer 3	2.5
100 mM $MgSO_4$	4.5
10 mM dNTPs	1.5
8 U/µl Bst Pol	0.6
10 U/µl N.BstNBI	1.5
20 µM Forward Template	0.25
20 µM Reverse Template	0.25
Total reaction mixture	47.5
Target sample	2.5
Total Reaction Volume	50 µL

5 The concentrations of components for the reaction conditions in this example are as follows:

Concentration	**Component**
45.7mM	Tris-HCl
13.9 mM	KCl
10 mM	$(NH_4)_2SO_4$
50 mM	NaCl
0.5 mM	DTT
15 mM	$MgCl_2$
0.10%	Triton X-100
0.008 mM	EDTA
6 µg/mL	BSA
3.90%	Glycerol (can be lower if using a more concentrated enzyme stock)
0.3 U/uL	Nt.BstNBI
0.1-0.4 U/µL	Bst polymerase (large fragment)
0.1 µM	Forward template
0.1 µM	Reverse template

Variations in buffer conditions, $MgSO_4$ concentration, polymerase concentration, and template concentrations all can be optimized based on the assay sequence and desired detection method. The amount of glycerol may, for example, be lowered if a more concentrated enzyme stock is used. Also, those of ordinary skill in the art recognize that the reaction may be run
5 without EDTA or BSA; these components may be present in the reaction as part of the storage buffers for the enzymes. The volumes can be scaled for larger or smaller total reaction volumes. The volume is usually between 5 µL and 100 µL.

The template concentrations are typically in excess of the concentration of target. The concentrations of the forward and reverse templates can be at the same or at different
10 concentrations to bias the amplification of one product over the other. The concentration of each is usually between 10 nM and 1µM.

Additives such as BSA, non-ionic detergents such as Triton X-100 or Tween-20, DMSO, DTT, and RNase inhibitor may be included for optimization purposes without adversely affecting the amplification reaction.

15

Preparing/Adding Target

Targets may be diluted in 1 x Thermopol Buffer II, 1 x TE (pH 7.5) or H_20. Hot start conditions allow for faster, more specific amplification. In this case, the reaction mix (minus either enzymes or templates and target) is heated to the reaction temperature for 2 minutes, after
20 which the reaction mix is added to the other component (enzymes or templates/target). The target can be added in any volume up to the total amount of water required in the reaction. In this case, the target would be diluted in water. In the example above for a 50 µL total reaction volume, 2.5 µL of the prepared target should be added per reaction to bring the total reaction volume to 50 µL.

25

Running the Reaction

The reaction is run at a constant temperature, usually between 54°C and 60°C for the enzyme combination of Bst polymerase (large fragment) and Nt.Bst.NB1 nicking enzyme. Other enzyme combinations may be used and the optimal reaction temperature will be based on the
30 optimal temperature for both the nicking enzyme and polymerase to work in concert as well as the melting temperature of the reaction products. The reaction is held at temperature for 2.5 to 10

minutes until the desired amount of amplification is achieved. The reaction may be stopped by either a heat denaturation step to denature the enzymes (when using enzymes that can be heat-killed). Alternatively, the reaction may be stopped by adding EDTA to the reaction.

5 Readout

The amplified target sequence may be detected by any method known to one of ordinary skill in the art. By way of non-limiting example, several of these known methods are presented herein. In one method, amplified products may be detected by gel electrophoresis, thus detecting reaction products having a specific length. The nucleotides may, for example, be labeled, such
10 as, for example, with biotin. Biotin-labeled amplified sequences may be captured using avidin bound to a signal generating enzyme, for example, peroxidase.

Nucleic acid detection methods may employ the use of dyes that specifically stain double-stranded DNA. Intercalating dyes that exhibit enhanced fluorescence upon binding to DNA or RNA are a basic tool in molecular and cell biology. Dyes may be, for example, DNA or
15 RNA intercalating fluorophores and may include but are not limited to the following examples: Acridine orange, ethidium bromide, Hoechst dyes, PicoGreen, propidium iodide, SYBR I (an asymmetrical cyanine dye), SYBR II, TOTO (a thiaxole orange dimer) and YOYO (an oxazole yellow dimer). Dyes provide an opportunity for increasing the sensitivity of nucleic acid detection when used in conjunction with various detection methods and may have varying
20 optimal usage parameters. For example ethidium bromide is commonly used to stain DNA in agarose gels after gel electrophoresis and during PCR (Hiquchi et al., Nature Biotechnology 10; 413-417, April 1992), propidium iodide and Hoechst 33258 are used in flow cytometry to determine DNA ploidy of cells, SYBR Green 1 has been used in the analysis of double-stranded DNA by capillary electrophoresis with laser induced fluorescence detection and Pico Green has
25 been used to enhance the detection of double-stranded DNA after matched ion pair polynucleotide chromatography (Singer et al., Analytical Biochemistry 249, 229-238 1997).

Nucleic acid detection methods may also employ the use of labeled nucleotides incorporated directly into the target sequence or into probes containing complementary sequences to the target of interested. Such labels may be radioactive and/or fluorescent in nature
30 and can be resolved in any of the manners discussed herein.

Methods of detecting and/or continuously monitoring the amplification of nucleic acid products are also well known to those skilled in the art and several examples are described below.

The production or presence of target nucleic acids and nucleic acid sequences may be detected and monitored by Molecular Beacons. Molecular Beacons are hair-pin shaped oligonucleotides containing a fluorophore on one end and a quenching dye on the opposite end. The loop of the hair-pin contains a probe sequence that is complementary to a target sequence and the stem is formed by annealing of complementary arm sequences located on either side of the probe sequence. A fluorophore and a quenching molecule are covalently linked at opposite ends of each arm. Under conditions that prevent the oligonucleotides from hybridizing to its complementary target or when the molecular beacon is free in solution the fluorescent and quenching molecules are proximal to one another preventing fluorescence resonance energy transfer (FRET). When the molecular beacon encounters a target molecule, hybridization occurs; the loop structure is converted to a stable more rigid conformation causing separation of the fluorophore and quencher molecules leading to fluorescence (Tyagi et al. Nature Biotechnology 14: March 1996, 303-308). Due to the specificity of the probe, the generation of fluorescence is exclusively due to the synthesis of the intended amplified product.

Molecular beacons are extraordinarily specific and can discern a single nucleotide polymorphism. Molecular beacons can also be synthesized with different colored fluorophores and different target sequences, enabling several products in the same reaction to be quantitated simultaneously. For quantitative amplification processes, molecular beacons can specifically bind to the amplified target following each cycle of amplification, and because non-hybridized molecular beacons are dark, it is not necessary to isolate the probe-target hybrids to quantitatively determine the amount of amplified product. The resulting signal is proportional to the amount of amplified product. This can be done in real time. As with other real time formats, the specific reaction conditions must be optimized for each primer/probe set to ensure accuracy and precision.

The production or presence of target nucleic acids and nucleic acid sequences may also be detected and monitored by Fluorescence resonance energy transfer (FRET). FRET is an energy transfer mechanism between two chromophores: a donor and an acceptor molecule. Briefly, a donor fluorophore molecule is excited at a specific excitation wavelength. The

subsequent emission from the donor molecule as it returns to its ground state may transfer excitation energy to the acceptor molecule through a long range dipole-dipole interaction. The intensity of the emission of the acceptor molecule can be monitored and is a function of the distance between the donor and the acceptor, the overlap of the donor emission spectrum and the
5 acceptor absorption spectrum and the orientation of the donor emission dipole moment and the acceptor absorption dipole moment. FRET is a useful tool to quantify molecular dynamics, for example, in DNA-DNA interactions as seen with Molecular Beacons. For monitoring the production of a specific product a probe can be labeled with a donor molecule on one end and an acceptor molecule on the other. Probe-target hybridization brings a change in the distance or
10 orientation of the donor and acceptor and FRET change is observed. (Joseph R. Lakowicz, "Principles of Fluorescence Spectroscopy", Plenum Publishing Corporation, 2nd edition (July 1, 1999)).

The production or presence of target nucleic acids and nucleic acid sequences may also be detected and monitored by Mass Spectrometry. Mass Spectrometry is an analytical technique
15 that may be used to determine the structure and quantity of the target nucleic acid species and can be used to provide rapid analysis of complex mixtures. Following the method, samples are ionized, the resulting ions separated in electric and/or magnetic fields according to their mass-to-charge ratio, and a detector measures the mass-to-charge ratio of ions. (Crain, P. F. and McCloskey, J. A., Current Opinion in Biotechnology 9: 25-34 (1998)). Mass spectrometry
20 methods include, for example, MALDI, MALDI/TOF, or Electrospray. These methods may be combined with gas chromatography (GC/MS) and liquid chromatography (LC/MS). MS has been applied to the sequence determination of DNA and RNA oligonucleotides (Limbach P., MassSpectrom. Rev. 15: 297-336 (1996); Murray K., J. Mass Spectrom. 31: 1203-1215 (1996)). MS and more particularly, matrix-assisted laser desorption/ionization MS (MALDI MS) has the
25 potential of very high throughput due to high-speed signal acquisition and automated analysis off solid surfaces. It has been pointed out that MS, in addition to saving time, measures an intrinsic property of the molecules, and therefore yields a significantly more informative signal (Koster H. et al., Nature Biotechnol., 14: 1123-1128 (1996)).

The production or presence of target nucleic acids and nucleic acid sequences may also
30 be detected and monitored by various methods of gel electrophoresis. Gel electrophoresis involves the separation of nucleic acids through a matrix, generally a cross-linked polymer, using

an electromotive force that pulls the molecules through the matrix. Molecules move through the matrix at different rates causing a separation between products that can be visualized and interpreted via any one of a number of methods including but not limited to; autoradiography, phosphorimaging, and staining with nucleic acid chelating dyes.

5 The production or presence of target nucleic acids and nucleic acid sequences may also be detected and monitored by capillary gel electrophoresis. Capillary-gel Electrophoresis (CGE) is a combination of traditional gel electrophoresis and liquid chromatography that employs a medium such as polyacrylamide in a narrow bore capillary to generate fast, high-efficient separations of nucleic acid molecules with up to single base resolution. CGE is commonly 10 combined with laser induced fluorescence (LIF) detection where as few as six molecules of stained DNA can be detected. CGE/LIF detection generally involves the use of fluorescent DNA intercalating dyes including ethidium bromide, YOYO and SYBR Green 1 but can also involve the use of fluorescent DNA derivatives where the fluorescent dye is covalently bound to the DNA. Simultaneous identification of several different target sequences can be made using this 15 method.

The production or presence of target nucleic acids and nucleic acid sequences may also be detected and monitored by various surface capture methods. This is accomplished by the immobilization of specific oligonucleotides to a surface producing a biosensor that is both highly sensitive and selective. Surfaces used in this method may include but are not limited to gold and 20 carbon and may use a number of covalent or noncovalent coupling methods to attach the probe to the surface. The subsequent detection of a target DNA can be monitored by a variety of methods.

Electrochemical methods generally involve measuring the cathodic peak of intercalators, such as methylene blue, on the DNA probe electrode and visualized with square wave 25 voltammograms. Binding of the target sequence can be observed by a decrease in the magnitude of the voltammetric reduction signals of methylene blue as it interacts with dsDNA and ssDNA differently reflecting the extent of the hybrid formation.

Surface Plasmon Resonance (SPR) can also be used to monitor the kinetics of probe attachment as well as the process of target capture. SPR does not require the use of fluorescence 30 probes or other labels. SPR relies on the principle of light being reflected and refracted on an interface of two transparent media of different refractive indexes. Using monochromatic and p-

polarized light and two transparent media with an interface comprising a thin layer of gold, total reflection of light is observed beyond a critical angle, however the electromagnetic field component of the light penetrates into the medium of lower refractive index creating an evanescent wave and a sharp shadow (surface plasmon resonance). This is due to the resonance 5 energy transfer between the wave and the surface plasmons. The resonance conditions are influenced by the material absorbed on the thin metal film and nucleic acid molecules, proteins and sugars concentrations are able to be measured based on the relation between resonance units and mass concentration.

The production or presence of target nucleic acids and nucleic acid sequences may also 10 be detected and monitored by lateral flow devices. Lateral Flow devices are well known. These devices generally include a solid phase fluid permeable flow path through which fluid flows through by capillary force. Examples include, but are not limited to, dipstick assays and thin layer chromatographic plates with various appropriate coatings. Immobilized on the flow path are various binding reagents for the sample, binding partners or conjugates involving binding 15 partners for the sample and signal producing systems. Detection of samples can be achieved in several manners; enzymatic detection, nanoparticle detection, colorimetric detection, and fluorescence detection, for example. Enzymatic detection may involve enzyme-labeled probes that are hybridized to complementary nucleic aid targets on the surface of the lateral flow device. The resulting complex can be treated with appropriate markers to develop a readable signal. 20 Nanoparticle detection involves bead technology that may use colloidal gold, latex and paramagnetic nanoparticles. In one example, beads may be conjugated to an anti-biotin antibody. Target sequences may be directly biotinylated, or target sequences may be hybridized to a sequence specific biotinylated probes. Gold and latex give rise to colorimetric signals visible to the naked eye and paramagnetic particles give rise to a non-visual signal when excited 25 in a magnetic field and can be interpreted by a specialized reader.

Fluorescence-based lateral flow detection methods are also known, for example, dual fluorescein and biotin-labeled oligo probe methods, UPT-NALF utilizing up-converting phosphor reporters composed of lanthanide elements embedded in a crystal (Corstjens et al., Clinical Chemistry, 47:10, 1885-1893, 2001), as well as the use of quantum dots.

30 Nucleic acids can also be captured on lateral flow devices. Means of capture may include antibody dependent and antibody independent methods. Antibody-dependent capture generally

comprises an antibody capture line and a labeled probe of complementary sequence to the target. Antibody-independent capture generally uses non-covalent interactions between two binding partners, for example, the high affinity and irreversible linkage between a biotinylated probe and a streptavidin line. Capture probes may be immobilized directly on lateral flow membranes. Both antibody dependent and antibody independent methods may be used in multiplexing.

The production or presence of target nucleic acids and nucleic acid sequences may also be detected and monitored by multiplex DNA sequencing. Multiplex DNA sequencing is a means of identifying target DNA sequences from a pool of DNA. The technique allows for the simultaneous processing of many sequencing templates. Pooled multiple templates can be resolved into individual sequences at the completion of processing. Briefly, DNA molecules are pooled, amplified and chemically fragmented. Products are fractionated by size on sequencing gels and transferred to nylon membranes. The membranes are probed and autoradiographed using methods similar to those used in standard DNA sequencing techniques (Church et al., Science 1998 Apr 8;240(4849):185-188). Autoradiographs can be evaluated and the presence of target nucleic acid sequence can be quantitated.

Kits

Kits of the present invention may comprise, for example, one or more polymerases, forward and reverse templates, and one or more nicking enzymes, as described herein. Where one target is to be amplified, one or two nicking enzymes may be included in the kit. Where multiple target sequences are to be amplified, and the templates designed for those target sequences comprise the nicking enzyme sites for the same nicking enzyme, then one or two nicking enzymes may be included. Or, where the templates are recognized by different nicking enzymes, more nicking enzymes may be included in the kit, such as, for example, 3 or more.

The kits of the present invention may also comprise one or more of the components in any number of separate containers, packets, tubes, vials, microtiter plates and the like, or the components may be combined in various combinations in such containers.

The components of the kit may, for example, be present in one or more containers, for example, all of the components may be in one container, or, for example, the enzymes may be in a separate container from the templates. The components may, for example, be lyophilized, freeze dried, or in a stable buffer. In one example, the polymerase and nicking enzymes are in

lyophilized form in a single container, and the templates are either lyophilized, freeze dried, or in buffer, in a different container. Or, in another example, the polymerase, nicking enzymes, and the templates are, in lyophilized form, in a single container. Or, the polymerase and the nicking enzyme may be separated into different containers.

5 Kits may further comprise, for example, dNTPs used in the reaction, or modified nucleotides, cuvettes or other containers used for the reaction, or a vial of water or buffer for re-hydrating lyophilized components. The buffer used may, for example, be appropriate for both polymerase and nicking enzyme activity.

The kits of the present invention may also comprise instructions for performing one or 10 more methods described herein and/or a description of one or more compositions or reagents described herein. Instructions and/or descriptions may be in printed form and may be included in a kit insert. A kit also may include a written description of an Internet location that provides such instructions or descriptions.

Kits may further comprise reagents used for detection methods, such as, for example, 15 reagents used for FRET, lateral flow devices, dipsticks, fluorescent dye, colloidal gold particles, latex particles, a molecular beacon, or polystyrene beads.

Examples

Example 1: Detection of DNA NEAR assay products by gel electrophoresis

20 The NEAR amplification reaction products can be visualized by gel electrophoresis. In the absence of target, the templates (with complementary 3′ bases) overlap by one or more bases, polymerase extends in each direction to generate the NEAR amplification duplex (Figure 1B); and the amplification proceeds in a similar mechanism to the NEAR amplification to amplify a product that is two bases shorter than the target amplified product. In the case of a 25mer assay 25 where the templates end in A and T, the resulting background product is 23 bases. The 27mer assay also forms a 23mer background and 27mer product. Longer reaction products are also amplified. The sequence of these products is hypothesized to be due to the polymerase extension before the nicking enzyme can nick both sides of the NEAR amplification duplex, according to Steps 9B in Figure 1C. Figure 2 shows the NEAR reaction products are easily distinguished from 30 background products by gel electrophoresis.

Example 2: Detection of RNA NEAR assay products by gel electrophoresis

The NEAR reaction can also amplify RNA targets. In this case, the target is Ebola Armored RNA, which is a ~600 base strand of RNA encapsulated by MS2 phage coat proteins to simulate a viral particle. The reaction is designed to amplify a 25-base region of the Ebola genome contained within the encapsulated RNA sequence. Reaction products run on a 20% polyacrylamide gel (Figure 3) show the amplified 25mer product along with 23mer and 20mer background products. This example demonstrates the ability of the NEAR reaction to amplify RNA released from virus-like particles.

Example 3: Detection of DNA and RNA NEAR assay products by mass spectrometry

The NEAR reaction amplification products can also be detected by mass spectrometry using an ESI/TOF system with a front end LC. The reaction products observed are multiple charged ion species. Usually, the -3 or -4 charge state is the major peak in the spectrum (in the range of 1000-3000 AMU), depending on the length of the oligonucleotide product. The sodium adduct is usually present in the spectrum as a peak adjacent to the major peak at roughly 20-25% the intensity. The unique peaks for the positive reactions in the presence of target are visible in both Figures 4 and 5 for the DNA and RNA NEAR reactions respectively. The background products formed in these NEAR reactions are not shown in the mass range of these spectra.

Example 4: Real-time detection of the NEAR assay amplification

The NEAR amplification reaction can also be monitored, as shown in Figure 6, in real-time with SYBR II fluorescence. The fluorescence increases as SYBR II intercalates into the amplified double-stranded products. The background products also generate fluorescence at a slower rate than the true product. Optimization of amplification sequence, reaction temperature and reaction buffer conditions are necessary in order to visualize distinct separation between the positive reactions and the negative controls.

Example 5: FRET detection of real-time NEAR assay amplification

NEAR amplification can also be monitored by Fluorescence Resonance Energy Transfer (FRET), as shown in Figure 7. Amplification occurs using dual labeled templates, one on each end (5′-FAM, 3′-BHQ). Fluorescence is generated from the FAM-labeled oligonucleotide upon

cleavage of the template by the nicking enzyme when it becomes double-stranded. Since fluorescence is produced by the initial nicking reaction, this detection method is extremely responsive. Since the 3′ ends of the templates are blocked from extension by the quenching label, the production of background fluorescence is inhibited.

5

Example 6: Molecular beacon detection of real-time NEAR amplification

A third method of monitoring real-time amplification is using molecular beacons, as shown in Figure 8. In this case, the amplified product hybridizes to the loop region of the molecular beacon resulting in an increase in fluorescence from the separation of the fluorophore
10 and quencher on each end of the hairpin stem. Since this interaction occurs post-amplification, it is considered pseudo-real-time and can be slightly slower in response relative to the FRET approach.

Example 7: False Alarm Rate testing

15 This experiment was designed to probe the probability that the NEAR amplification reaction will yield a true product in the negative reaction, or a false positive. NEAR reactions directed at specific amplification of a 25mer region specific to the *Bacillus subtilis* genome were run in the presence (n = 120) and absence (n = 320) of *Bacillus subtilis* genomic DNA. End point reactions were run on the mass spectrometer and the area under the curve (AUC) for the product
20 mass peak in the mass spectrum was analyzed. As shown in Figure 9, the results show that none of the 320 negative reactions resulted in a false positive with AUC values equal to the water control. The true positive AUC values were at least 3 standard deviations apart from the true negatives. Overall, these results demonstrate the reproducible nature of the NEAR assay.

25 **Example 8:** Beacon detection: NEAR assay reproducibility with beacon detection

The molecular beacon detection of NEAR reaction products can also be used as an endpoint reading. As shown in Figure 10, the ratio of NEAR reaction products can be manipulated by varying the input ratio of the forward and reverse templates. Skewing the templates to favor one of the reaction products allows the single-stranded product to be available
30 for hybridization to a molecular beacon. The open beacon generates a fluorescent signal. This detection method is extremely reproducible. In this study, two operators performed replicates of

the same assay on two different days. The results of this study demonstrate the reproducibility of the assay from one day to the next as well as reproducibility between operators.

Example 9: NEAR Assay sensitivity with beacon detection

5 The sensitivity of the NEAR assay with beacon read-out was tested using a dilution of *Francisella tularensis* genomic DNA. As shown in Figure 11, as few as 50 copies were detected above the no target control.

Example 10: Concentration of amplified products for NEAR DNA amplification

10 The sensitivity of the NEAR assay has also been studied using mass spectrometry detection of the reaction products. Figure 12 shows signal above the no target control down to 100 copies. The data from this study was used to correlate the input copy number to the final amount of amplified product. In this study, the AUC values of the mass spec product peaks were fit to a standard curve to give the estimated final concentration of amplified product for the
15 NEAR assay. The amount of amplified product ranges from approximately 250nM to almost 1μM for 1E+2 and 1E+5 copies respectively. This product amount results in a 1E+8 to 7E+10-fold amplification. These reactions were performed without the hot-start conditions, in fact hot-start conditions have been shown to dramatically increase the amount of product amplified, so a further increase in amplification is achieved. The zero copy amplification reaction has a positive
20 final concentration due to the y-intercept value in the standard curve equation.

Example 11: Concentration of amplified products for RNA assay

A similar study was performed on the NEAR amplification of RNA. A dilution of RNA targets were amplified by the NEAR assay. Products were run on the mass spec and the AUC
25 values of the product peaks were analyzed against a standard curve to determine the concentration of the final product, as shown in Figure 13. A 12 minute amplification starting with 30 and 30,000 copies of initial target results in a 3E+9 to 1E+7 -fold amplification respectively. The lower extent of amplification compared to the DNA amplification could be due to the less efficient reverse transcriptase ability of the polymerase compared to its replication
30 abilities. Also, the RNA:DNA hybrid formed upon the extension of the reverse template is a stronger interaction compared to a normal DNA:DNA hybrid and will have less breathing to

allow for the forward or another reverse template to displace one strand. However, amplification products from the RNA reaction were detected down to <100 copies.

Example 12: NEAR reaction specificity for DNA

Since the reaction products are usually between 20 and 30 bases in length, the question arises as to whether or not these short amplification assays can be specific enough to target a single sequence region with other near neighbor genomes present. The NEAR reaction was tested for its specificity by running the amplification reaction in the presence and absence of varying amounts of the near neighbor genomic DNA (Figure 14). In this case, the assay detects a specific sequence in the pXO2 plasmid of *Bacillus anthracis* and the near neighbor genome is *Bacillus thuringiensis* (kurstaki). The reactions were analyzed by the AUC values for the product peaks. The figure below demonstrates that in the absence of the correct target (*Bacillus anthracis*), there is no true product amplified (the levels are so low that they are not visible on the scale of the graph). The amount of amplification of the positive reactions is consistent, with larger error bars for the 0 and 5E+5 copies of *Bacillus thuringiensis* (5E+4 copies of *Bacillus anthracis*) due to a single lower value for one of the triplicate runs. Overall the experiment demonstrates that the NEAR reaction is very specific to the target sequence when the assay is designed within a unique region of the genome.

Example 13: Interferent testing

A panel of interferents was tested to monitor the effect of each on the NEAR assay amplification. Figure 15 demonstrates the robust nature of the NEAR assay in the presence of interferents. Some interferents that are known to inhibit PCR, such as humic acid, did not appear to inhibit the NEAR assay, though the amount of each interferent is unknown. From statistical analysis only interferent B, C, and E were statistically different from the control assay x. In the B, C, and E cases, the difference resulted in increased product amplification.

Example 14: Multiplexing of two sequences with NEAR DNA assays

A DNA duplex was designed for capillary electrophoresis (CE) detection. Amplification products were 25 bases (*Bacillus anthracis* assay, *Ba*) and 27 bases (*Bacillus subtilis* assay, *Bs*) in length with background production of a 23mer. The reaction was run for 10 minutes in the

presence or absence of 5E+5 copies of the respective genomic DNA target. The samples were run on a 20% polyacrylamide gel to visualize the reaction products. Figure 16 indicates the presence of positive product amplification when *Bacillus subtilis* only is present as well as when both *Bacillus subtilis* and *Bacillus anthracis* are present.

5

Example 15: NEAR DNA assay duplex specificity

The NEAR DNA duplex reaction with *Bacillus subtilis* (*Bs*) and *Bacillus anthracis* (*Ba*) was shown to be specific to the respective genomes. The assays were run in the presence of the near neighbor, *Bacillus thuringiensis*, as shown in Figure 17. In the negative reaction where
10 both template sets are present as well as the *Bacillus thuringiensis* genomic DNA, there is no product band in the 25 or 27mer region. Product bands appear only when the specific genomic target is present, which demonstrates the specificity of the duplex reaction.

Example 16: Multiplexing with NEAR RNA assays

15 An MS2 assay that amplifies a 27mer product and an Ebola assay that amplifies a 25mer product was developed and multiplexed so that all templates are present in each assay and amplification of products is dependent on the target present. This combination of templates forms background products that are 23 bases and 20 bases in length. The gel shown in Figure 18 demonstrates the ability for the NEAR reaction to amplify multiple RNA targets in a single
20 reaction.

Example 17: Amplification from lysed spores by NEAR Assay

Amplification was performed on semi-processed samples to determine whether it is possible to amplify DNA released from spores through lysis. The negative control reaction
25 contained DNase-treated spores, unlysed, so no DNA should be present to amplify. The positive control reaction contained purified genomic DNA at concentrations around the amount of DNA estimated to be released through lysis. Results in Figure 19 show that amplification with unlysed DNase-treated spores results in no product amplification as expected, whereas the three samples lysed before amplification resulted in product amounts in the range of the theoretical amounts.

30

Example 18: Capture and Extension

The NEAR reaction products can also be detected on a solid surface. A capture probe attached at the 5′ end to the surface through a biotin/streptavidin attachment can bind to the reaction products from which polymerase extends to form a stable duplex that SYBR and any
5 intercalating dye can detect. The capture probe is designed to favor extension through binding to the true product over background products because the 3′ base of the capture probe is complementary to the middle spacer base in the product which is not present in either of the templates or the background products. Figure 20 demonstrates the increased fluorescence of the NEAR products in the presence of the capture probe and polymerase over the average binding
10 (same reaction in the absence of polymerase, to preclude extension of the capture probe) and the no target control where only background products are amplified, but cannot form a stable duplex with the capture probe for polymerase to extend.

Example 19: Surface NEAR FRET DNA Assay

15 The NEAR reaction can also be performed with the templates immobilized on the surface. The templates for FRET detection of surface amplification usually have three modifications: one 5′ biotin with a TEG spacer, one FAM fluorophore internal to the biotin, and a quencher on the 3′ end which serves to block background amplification as well as to quench the FAM fluorophore. The template is immobilized on the surface through biotin/streptavidin
20 attachment. Figure 21 demonstrates that with both templates immobilized along with additional mixing, the reaction proceeds at a much slower rate than the solution amplification rate (amplification in 16 minutes for 1E+6 copies of genomic DNA). When a single template is immobilized on the surface and the other template is free in solution, the amplification reaction is increased to 10 minute detection for 1E+6 copies of genomic DNA. Fluorescence from
25 background products is observed ~3.5 minutes after the product signal, similar to what is observed for solution phase kinetics, but slowed considerably.

* * * * * * * *

The entirety of each patent, patent application, publication and document referenced
30 herein hereby is incorporated by reference. Citation of the above patents, patent applications,

publications and documents is not an admission that any of the foregoing is pertinent prior art, nor does it constitute any admission as to the contents or date of these publications or documents.

Singular forms "a", "an", and "the" include plural reference unless the context clearly dictates otherwise. Thus, for example, reference to "a subset" includes a plurality of such subsets, reference to "a nucleic acid" includes one or more nucleic acids and equivalents thereof known to those skilled in the art, and so forth. The term "or" is not meant to be exclusive to one or the terms it designates. For example, as it is used in a phrase of the structure "A or B" may denote A alone, B alone, or both A and B.

Unless defined otherwise, all technical and scientific terms used herein have the same meanings as commonly understood by one of ordinary skill in the art to which this invention belongs. Although any methods and systems similar or equivalent to those described herein can be used in the practice or testing of the present invention, the methods, devices, and materials are now described. All publications mentioned herein are incorporated herein by reference for the purpose of describing and disclosing the processes, systems, and methodologies that are reported in the publications which might be used in connection with the invention. Nothing herein is to be construed as an admission that the invention is not entitled to antedate such disclosure by virtue of prior invention.

Modifications may be made to the foregoing without departing from the basic aspects of the invention. Although the invention has been described in substantial detail with reference to one or more specific embodiments, those of ordinary skill in the art will recognize that changes may be made to the embodiments specifically disclosed in this application, and yet these modifications and improvements are within the scope and spirit of the invention. The invention illustratively described herein suitably may be practiced in the absence of any element(s) not specifically disclosed herein. Thus, for example, in each instance herein any of the terms "comprising", "consisting essentially of", and "consisting of" may be replaced with either of the other two terms. Thus, the terms and expressions which have been employed are used as terms of description and not of limitation, equivalents of the features shown and described, or portions thereof, are not excluded, and it is recognized that various modifications are possible within the scope of the invention. Embodiments of the invention are set forth in the following claims.

<u>What is claimed is</u>:

1. A method for amplifying a double-stranded nucleic acid target sequence, comprising
   a) contacting a target DNA molecule comprising a double-stranded target sequence, having a sense strand and an antisense strand, with a forward template and a reverse template, wherein
      i) said forward template comprises a nucleic acid sequence comprising a recognition region at the 3′ end that is complementary to the 3′ end of the target sequence antisense strand; a nicking enzyme site upstream of said recognition region and a stabilizing region upstream of said nicking enzyme site;
      ii) said reverse template comprises a nucleotide sequence comprising a recognition region at the 3′ end that is complementary to the 3′ end of the target sequence sense strand, a nicking enzyme site upstream of said recognition region, and a stabilizing region upstream of said nicking enzyme site;
   b) providing a first nicking enzyme that is capable of nicking at the nicking enzyme site of said forward template, and does not nick within said target sequence;
   c) providing a second nicking enzyme that is capable of nicking at the nicking enzyme site of said reverse template and does not nick within said target sequence; and
   d) providing a DNA polymerase;

under conditions wherein amplification is performed by multiple cycles of said polymerase extending said forward and reverse templates along said target sequence producing a double-stranded nicking enzyme site, and said nicking enzymes nicking at said nicking enzyme sites, producing an amplification product.

2. The method of claim 1, wherein said DNA polymerase is a thermophilic polymerase.

3. The method of claim 1, wherein said polymerase and said nicking enzymes are stable at temperatures up to 85 °C.

4. The method of claim 1, wherein said polymerase and said nicking enzymes are stable at temperatures up to 60 °C.

5. The method of claim 1, wherein said polymerase is selected from the group consisting of Bst (large fragment), 9°N, $Vent_R$® (exo-) DNA Polymerase, Therminator, and Therminator II.

6. The method of claim 1, wherein said nicking enzymes nick downstream of the nicking enzyme binding site.

5 7. The method of claim 1, wherein said forward and reverse templates comprise nicking enzyme sites recognized by the same nicking enzyme and said first and said second nicking enzyme are the same.

8. The method of claim 1 or 7, wherein said nicking enzymes are selected from the group consisting of Nt.BspQI, Nb.BbvCi, Nb.BsmI, Nb.BsrDI, Nb.BtsI, Nt.AlwI, Nt.BbvCI,
10 Nt.BstNBI, Nt.CviPII, Nb.Bpu10I, and Nt.Bpu10I.

9. The method of claim 1, wherein said target sequence comprises from 1 to 5 nucleotides more than the sum of the nucleotides of said forward template recognition region and said reverse template recognition region.

10. The method of claim 1, wherein the DNA molecule is genomic DNA.

15 11. The method of claim 1, wherein the DNA molecule is selected from the group consisting of plasmid, mitochondrial, and viral DNA.

12. The method of claim 1, wherein the forward template is provided at the same concentration as the reverse template.

13. The method of claim 1, wherein the forward template is provided at a ratio to the reverse
20 template at the range of ratios of 1:100 to 100:1

14. The method of claim 1, further comprising a second polymerase.

15. The method of claim 1, wherein the amplification is conducted at a constant temperature.

16. The method of claim 15, wherein the amplification is conducted between 54°C and 60°C.

17. The method of claim 15, wherein the amplification reaction is held at constant
25 temperature for 1 to 10 minutes.

18. The method of claim 1, further comprising detecting the amplification product.

19. The method of claim 18, wherein said amplification product is detected by a method selected from the group consisting of gel electrophoresis, mass spectrometry, SYBR I fluorescence, SYBR II fluorescence, SYBR Gold, Pico Green, TOTO-3, intercalating dye
30 detection, FRET, molecular beacon detection, surface capture, capillary electrophoresis,

incorporation of labeled nucleotides to allow detection by capture, fluorescence polarization, and lateral flow capture.

20. The method of claim 1, wherein at least two target sequences are capable of being amplified.

5 21. The method of claim 18, wherein said amplification products are detected on a solid surface.

22. The method of claim 1, wherein at least one capture probe is immobilized on a solid surface.

23. The method of claim 1, wherein at least one of said templates comprises a spacer,
10 blocking group, or a modified nucleotide.

24. A method for amplifying a single-stranded nucleic acid target sequence, comprising

a) contacting a target nucleic acid comprising a single-stranded target sequence with a reverse template, wherein said reverse template comprises a nucleotide sequence comprising a recognition region at the 3′ end that is complementary to
15 the 3′ end of the target sequence, a nicking enzyme site upstream of said recognition region, and a stabilizing region upstream of said nicking enzyme site;

b) providing a first nicking enzyme that is capable of nicking at the nicking enzyme site of said reverse template, and does not nick within said target sequence;

20 c) providing a DNA polymerase under conditions wherein said polymerase extends said reverse template along said target sequence;

d) contacting said extended reverse template with a forward template, wherein said forward template comprises comprising a recognition region at the 3′end that is identical to the 5′end of the target sequence a nicking enzyme site
25 upstream of said recognition region, and a stabilizing region upstream of said nicking enzyme site;

e) providing a second nicking enzyme that is capable of nicking at the nicking enzyme site of said forward template and does not nick within said target sequence;

30 under conditions wherein amplification is performed by multiple cycles of said polymerase extending said forward and reverse templates along said target sequence

producing a double-stranded nicking enzyme site, and said nicking enzymes nicking at said nicking enzyme sites, producing an amplification product.

25. The method of claim 24, wherein said DNA polymerase is a thermophilic polymerase.

26. The method of claim 24, wherein said polymerase and said nicking enzymes are stable at
5 temperatures up to 85 °C.

27. The method of claim 24, wherein said polymerase and said nicking enzymes are stable at temperatures up to 60 °C.

28. The method of claim 24, wherein said polymerase is selected from the group consisting of Bst (large fragment), 9°N, $Vent_R^{®}$ (exo-) DNA Polymerase, Therminator, and Therminator II.

10 29. The method of claim 24, wherein said nicking enzymes nick downstream of the nicking enzyme binding site.

30. The method of claim 24, wherein said forward and reverse templates comprise nicking enzyme sites recognized by the same nicking enzyme and said first and said second nicking enzyme are the same.

15 31. The method of claim 24 or 30, wherein said nicking enzymes are selected from the group consisting of Nt.BspQI, Nb.BbvCi, Nb.BsmI, Nb.BsrDI, Nb.BtsI, Nt.AlwI, Nt.BbvCI, Nt.BstNBI, Nt.CviPII, Nb.Bpu10I, and Nt.Bpu10I.

32. The method of claim 24, wherein said target sequence comprises from 1 to 5 nucleotides more than the sum of the nucleotides of said forward template recognition region and said
20 reverse template recognition region.

33. The method of claim 24, wherein the target sequence is RNA.

34. The method of claim 33, wherein the target sequence is messenger RNA.

35. The method of claim 33, wherein the target sequence is viral RNA.

36. The method of claim 33, wherein the target sequence is microRNA or a microRNA
25 precursor.

37. The method of claim 33, wherein the target sequence is microRNA.

38. The method of claim 33, wherein said polymerase comprises reverse transcription activity.

39. The method of claim 24, wherein the target sequence is DNA.

30 40. The method of claim 24, wherein the target sequence is genomic DNA.

41. The method of claim 24, wherein the target sequence is selected from the group consisting of plasmid, mitochondrial, and viral nucleic acid.

42. The method of claim 24, wherein the forward template is provided at the same concentration as the reverse template.

5 43. The method of claim 24, wherein the forward template is provided at a ratio to the reverse template at the range of ratios of 1:100 to 100:1

44. The method of claim 24, further comprising a second polymerase.

45. The method of claim 44, wherein at least one of said polymerases comprises reverse transcriptase activity.

10 46. The method of claim 24, wherein the amplification is conducted at a constant temperature.

47. The method of claim 46, wherein the amplification is conducted between 54°C and 60°C.

48. The method of claim 46, wherein the amplification reaction is held at constant temperature for 1 to 10 minutes.

15 49. The method of claim 24, further comprising detecting the amplification product.

50. The method of claim 49, wherein said amplification product is detected by a method selected from the group consisting of gel electrophoresis, mass spectrometry, SYBR I fluorescence, SYBR II fluorescence, SYBR Gold, Pico Green, TOTO-3, intercalating dye detection, FRET, molecular beacon detection, surface capture, capillary electrophoresis,
20 incorporation of labeled nucleotides to allow detection by capture, fluorescence polarization, and lateral flow capture.

51. The method of claim 24, wherein at least two target sequences are capable of being amplified.

52. The method of claim 49, wherein said amplification products are detected on a solid
25 surface.

53. The method of claim 49, wherein at least one capture probe is immobilized on a solid surface.

54. The method of claim 24, wherein at least one of said templates comprises a spacer, blocking group, or a modified nucleotide.

30 55. A set of oligonucleotide templates, comprising

a) a first template for nucleic acid amplification, comprising a recognition region at the 3′ end that is complementary to the 3′ end of a target sequence antisense strand; a nicking enzyme site upstream of said recognition region; and a stabilizing region upstream of said nicking enzyme site; and

5 b) a second template for nucleic acid amplification, comprising a recognition region at the 3′ end that is identical to the 5′ of said target sequence antisense strand; a nicking enzyme site upstream of said recognition region; and a stabilizing region upstream of said nicking enzyme site;

wherein said target sequence comprises from 1 to 5 spacer bases between said 3′ end of the

10 antisense strand and said 5′ end of said antisense strand that do not bind to either template.

56. A kit for amplifying a nucleic acid target sequence, comprising

a) a DNA polymerase;

b) a first template for nucleic acid amplification, comprising a recognition region at the 3′ end that is complementary to the 3′ end of a target sequence antisense strand; a

15 nicking enzyme site upstream of said recognition region; and a stabilizing region upstream of said nicking enzyme site;

c) a second template for nucleic acid amplification, comprising a recognition region at the 3′ end that is complementary to the 3′ end of a target sequence sense strand; a nicking enzyme site upstream of said recognition region; and a stabilizing region

20 upstream of said nicking enzyme site;

d) one or two thermostable nicking enzymes, wherein either one enzyme is capable of nicking at the nicking enzyme site of said first and said second templates, or a first enzyme is capable of nicking at the nicking enzyme site of said first primer and a second enzyme is capable of nicking at the enzyme site of said second primer.

25 57. The kit of claim 56, wherein said polymerase, nicking enzymes, and templates are in a container.

58. The kit of claim 56, wherein said polymerase, nicking enzymes, and templates are in two containers.

59. The kit of claim 56, wherein said polymerase and nicking enzymes are in a first

30 container, and said templates are in a second container.

60. The kit of claim 56, wherein said polymerase, nicking enzymes, and templates are lyophilized.

61. The kit of claim 56, further comprising instructions for following the method of amplification.

5 62. The kit of claim 56, further comprising a cuvette.

63. The kit of claim 56, further comprising a lateral flow device or dipstick.

64. The kit of claim 63, wherein said lateral flow device or dipstick further comprise a capture probe.

65. The kit of claim 56, further comprising a detector component selected from the group 10 consisting of a fluorescent dye, colloidal gold particles, latex particles, a molecular beacon, and polystyrene beads.

66. The kit of claim 56, wherein at least one of said templates comprises a spacer, blocking group, or a modified nucleotide.

15

Abstract

The invention is in general directed to the rapid exponential amplification of short DNA or RNA sequences at a constant temperature.

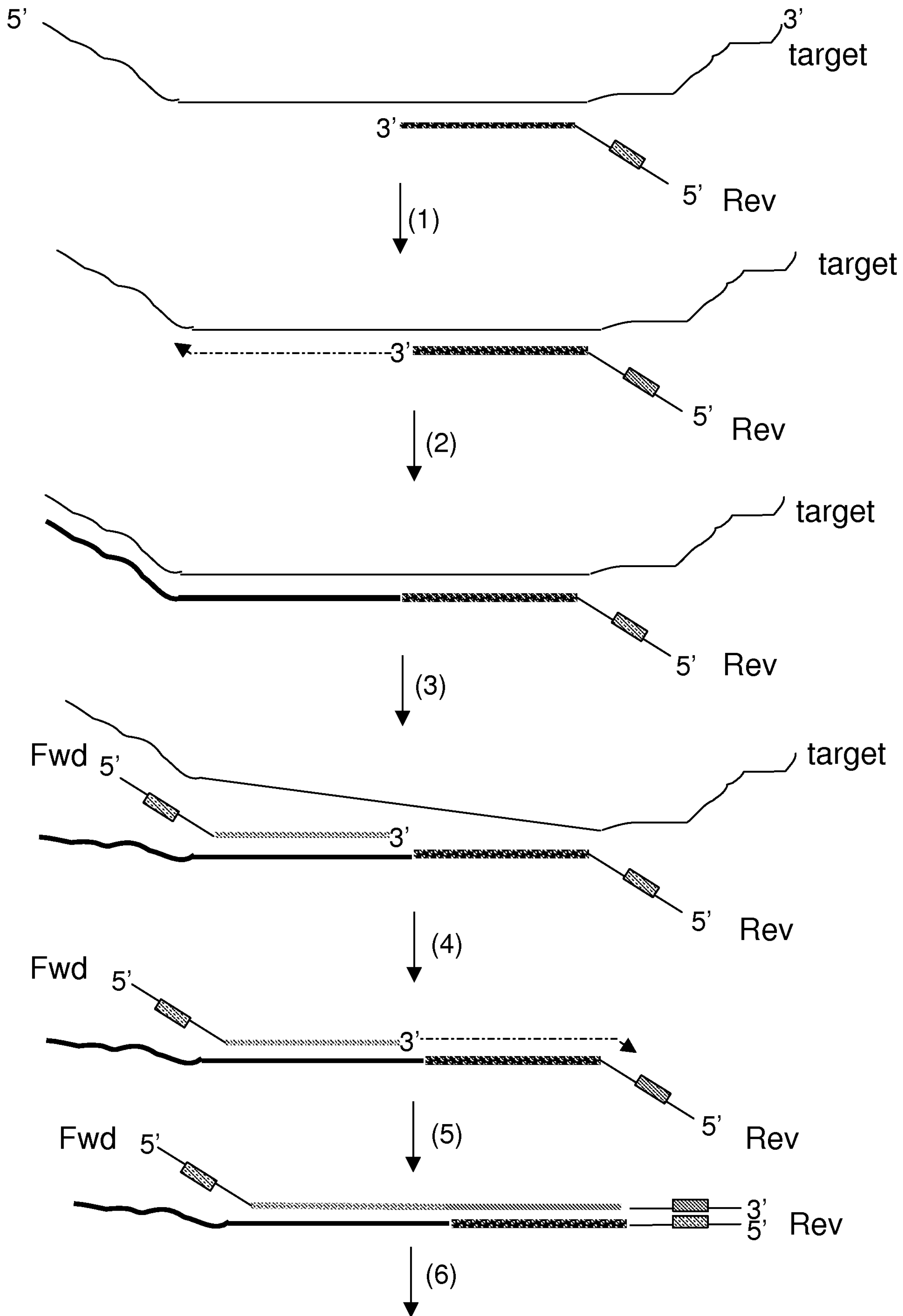


Figure 1A

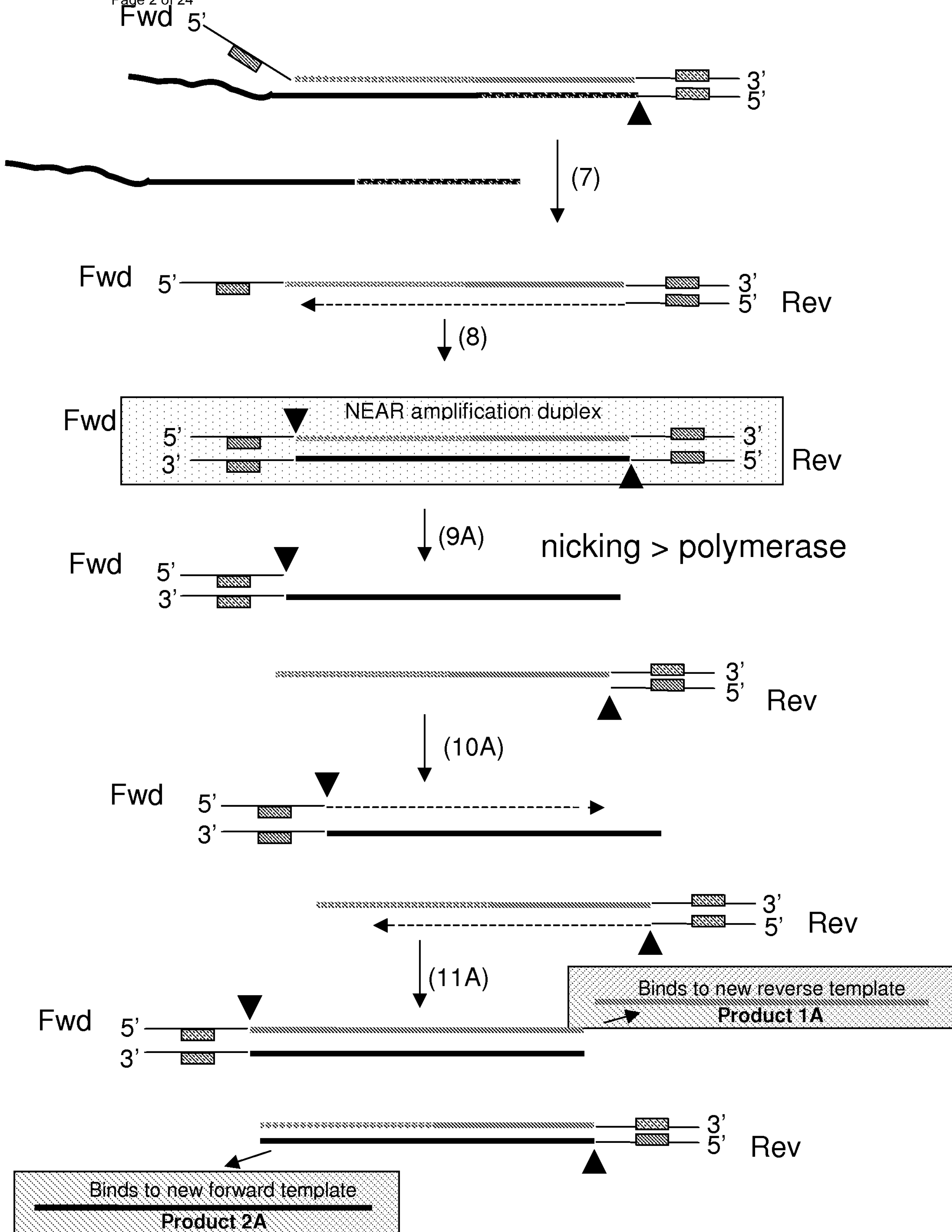


Figure 1B

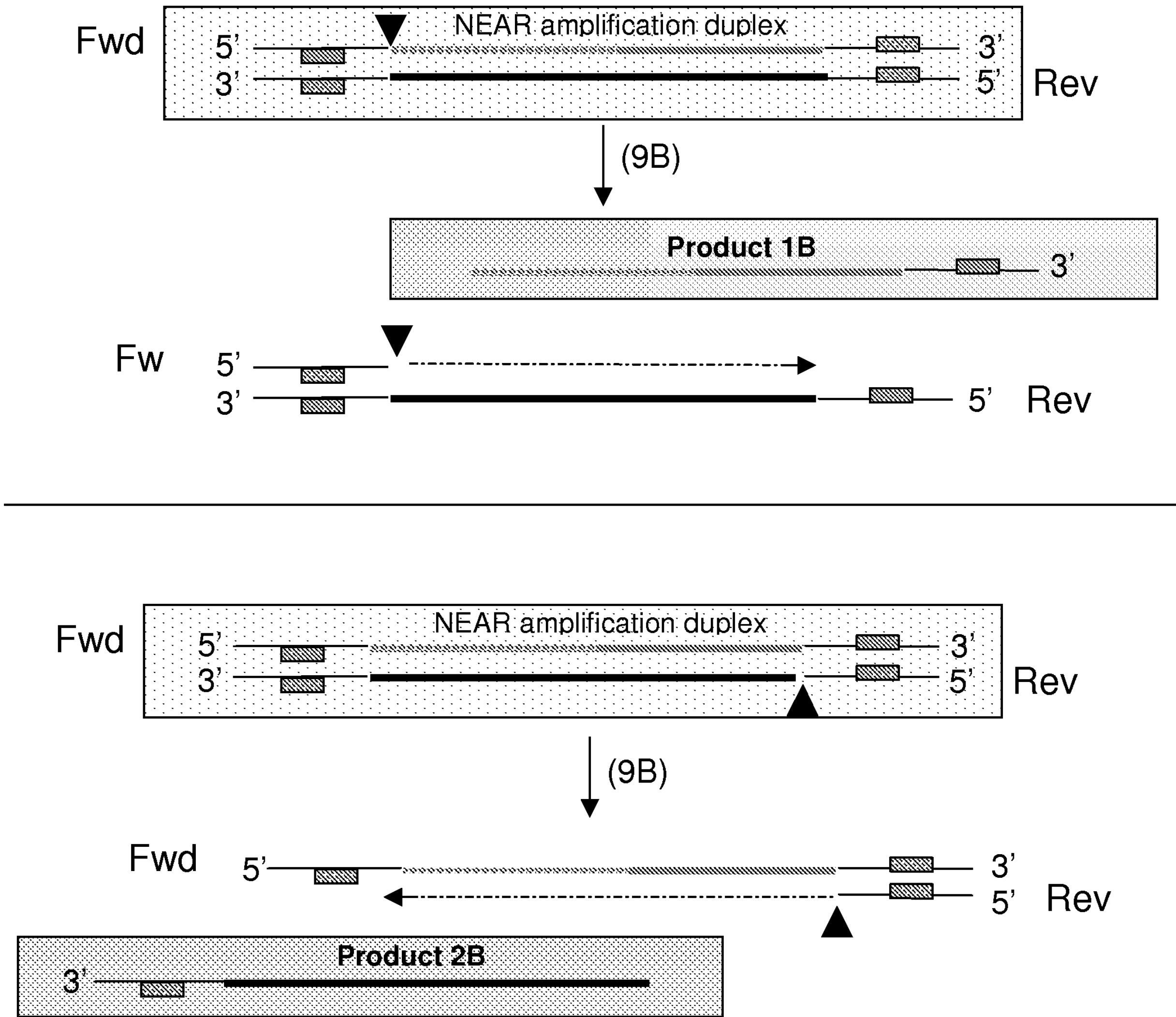
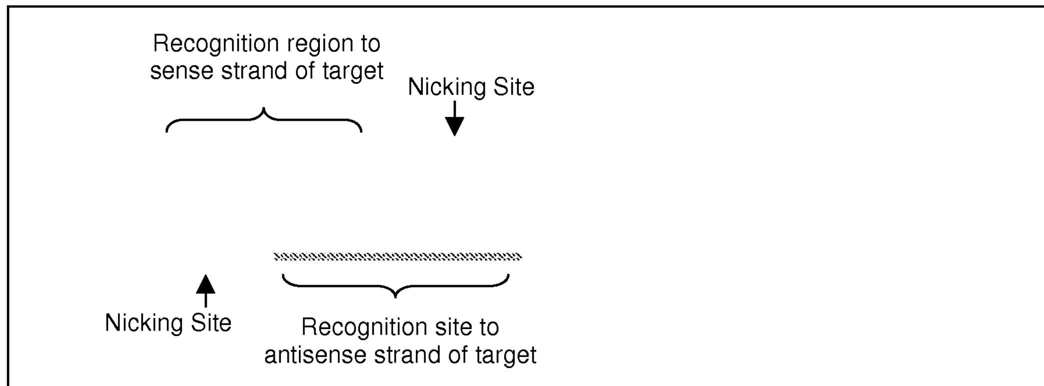


Figure 1C



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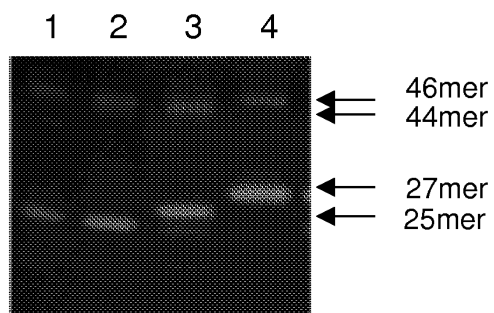

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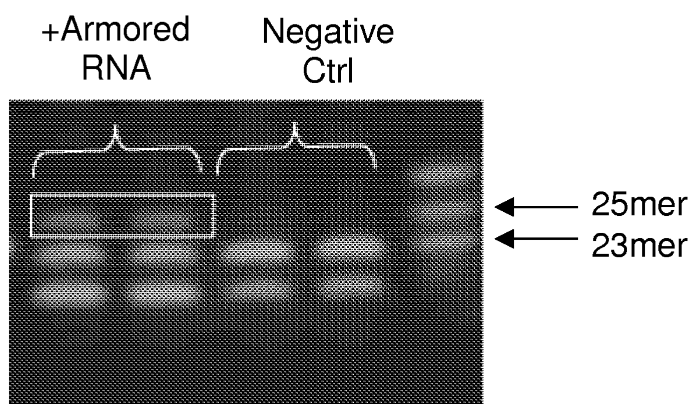
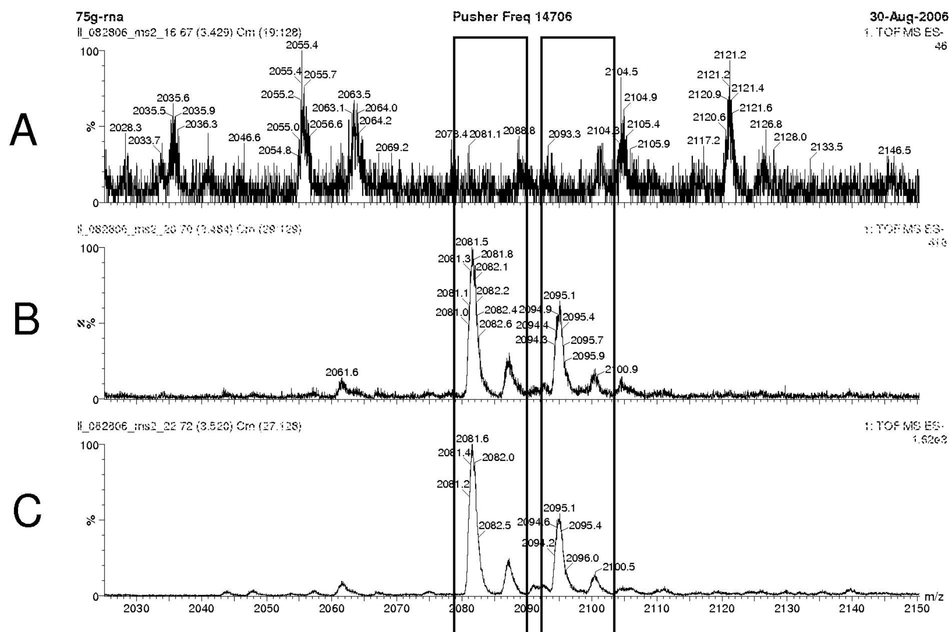


Figure 3

A

B

Figure 4



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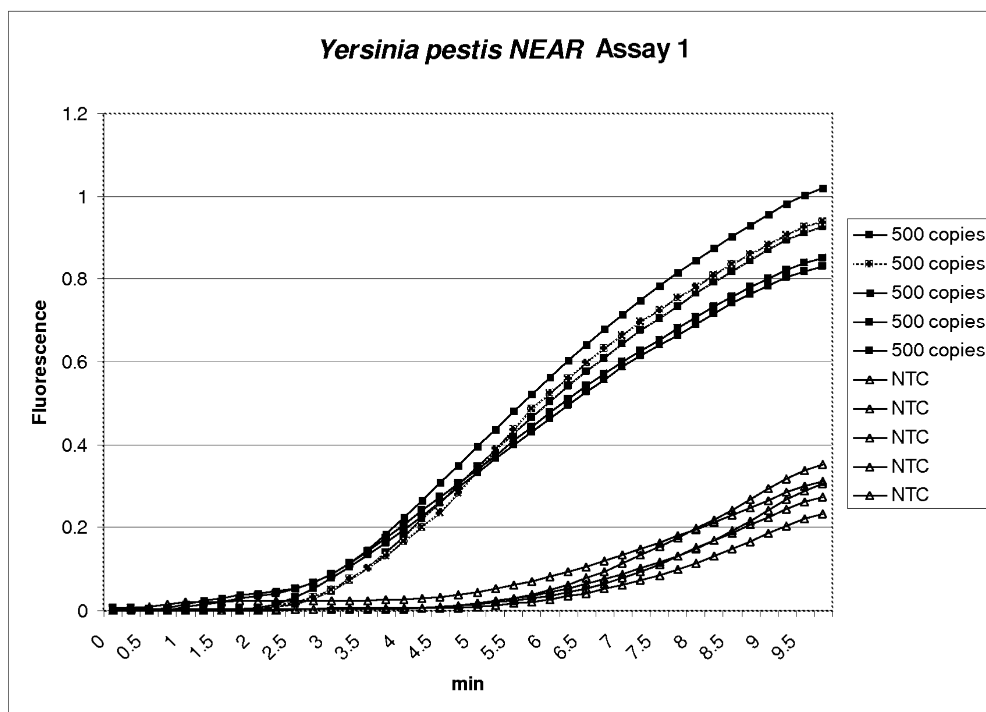

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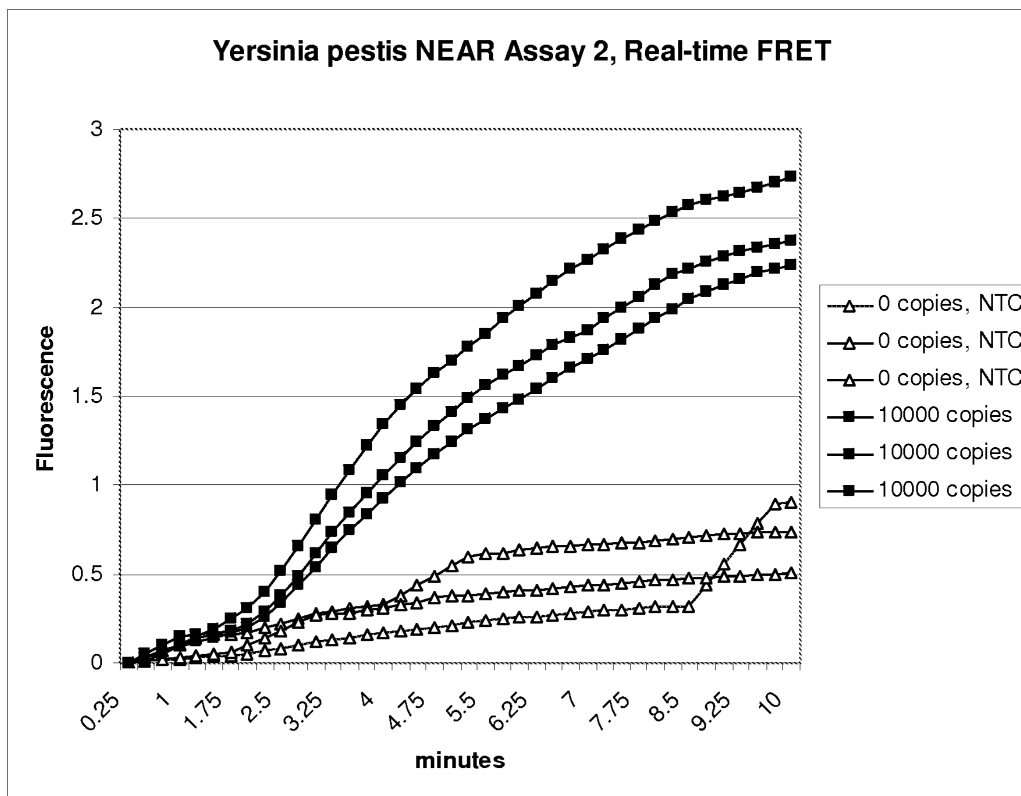

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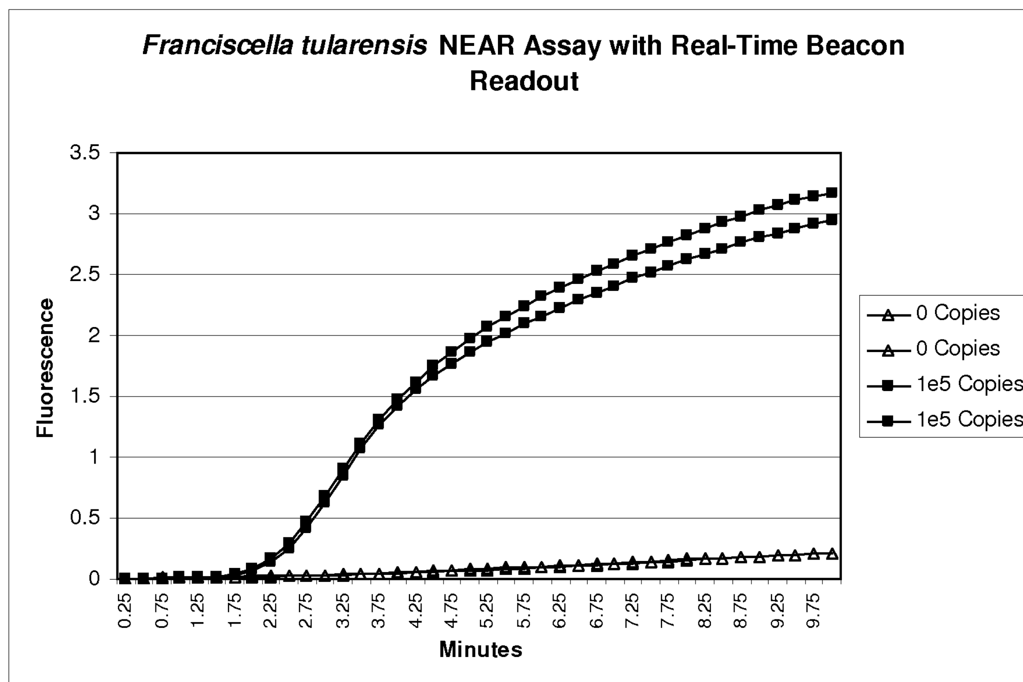

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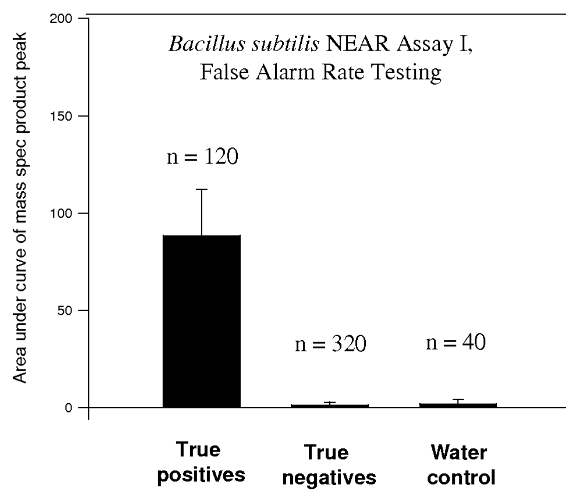

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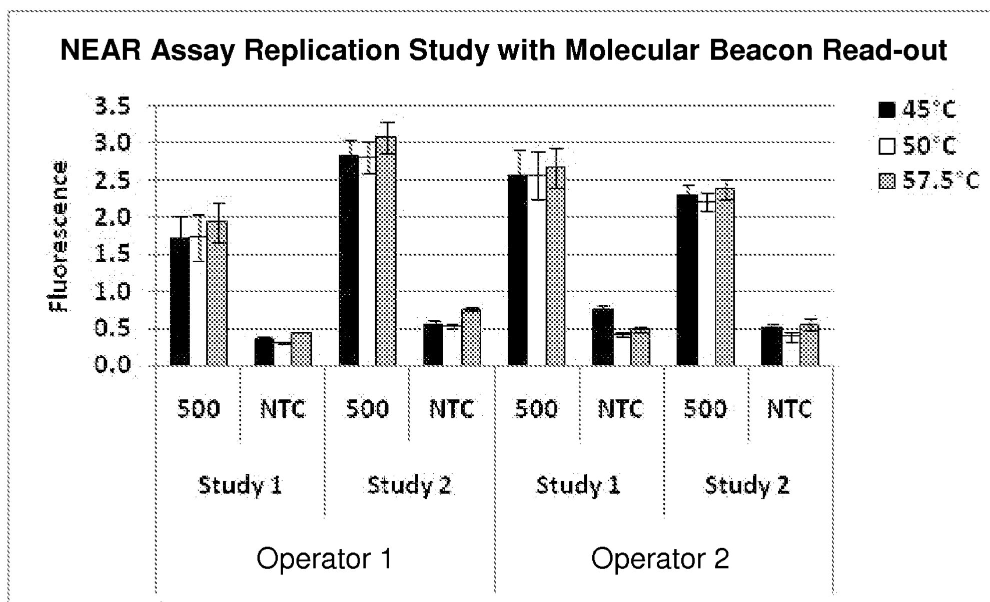

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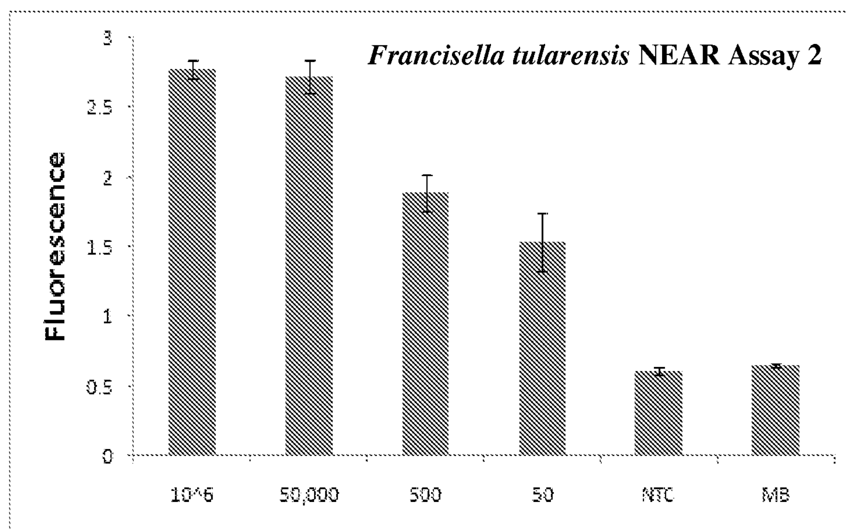

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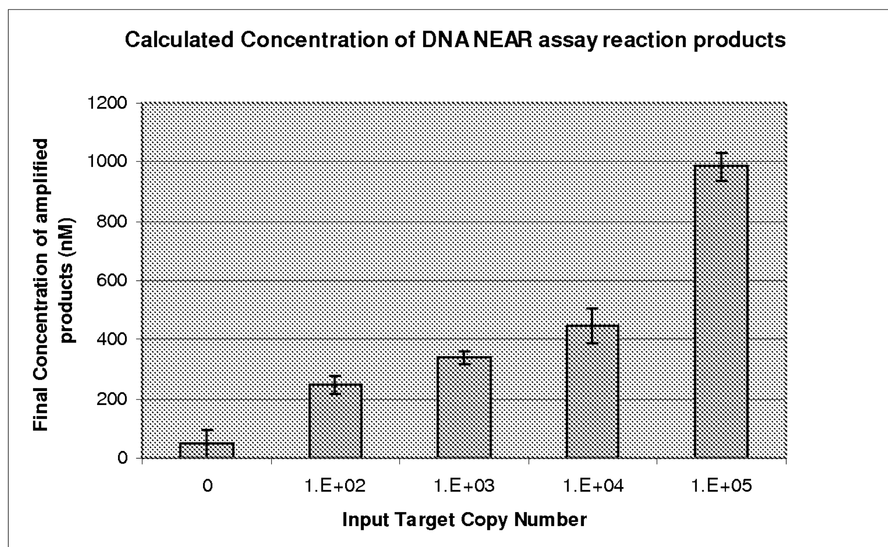

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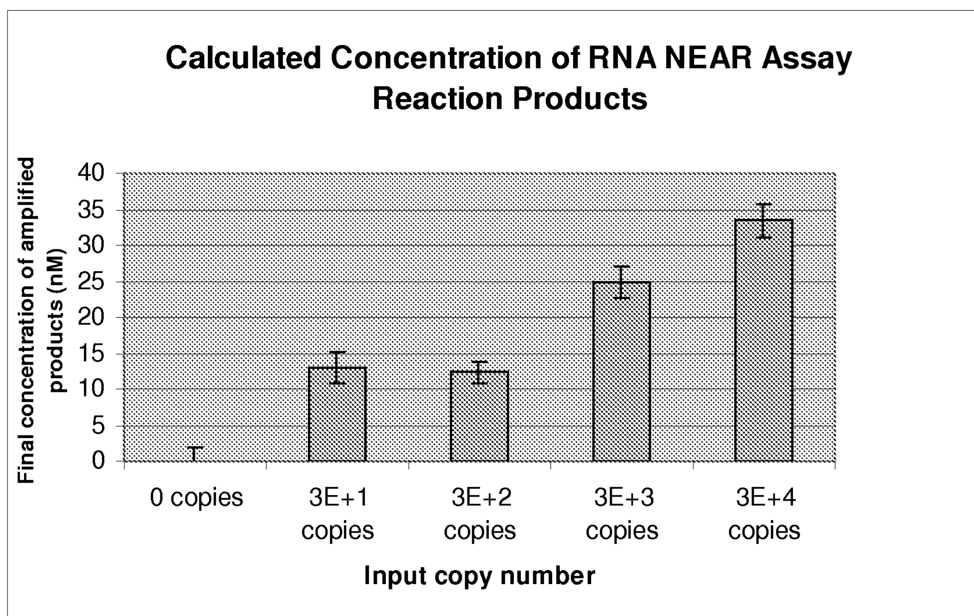

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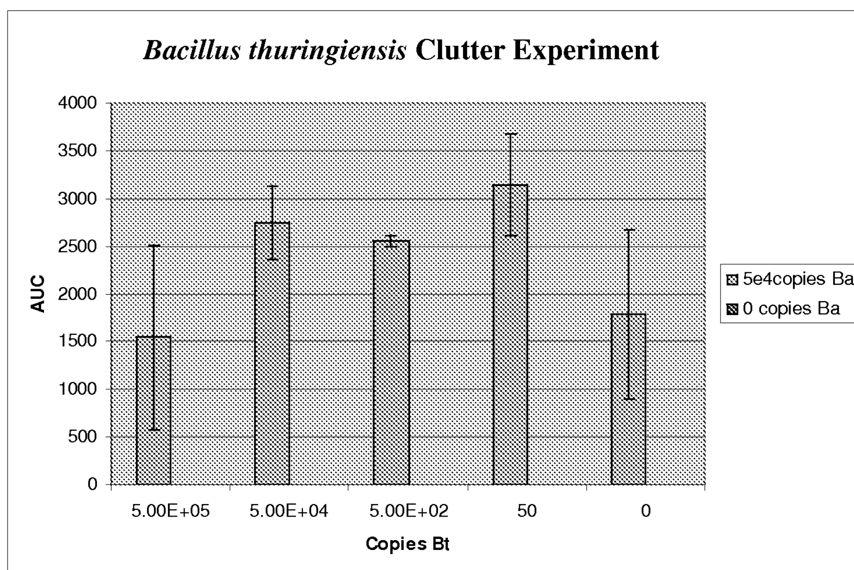

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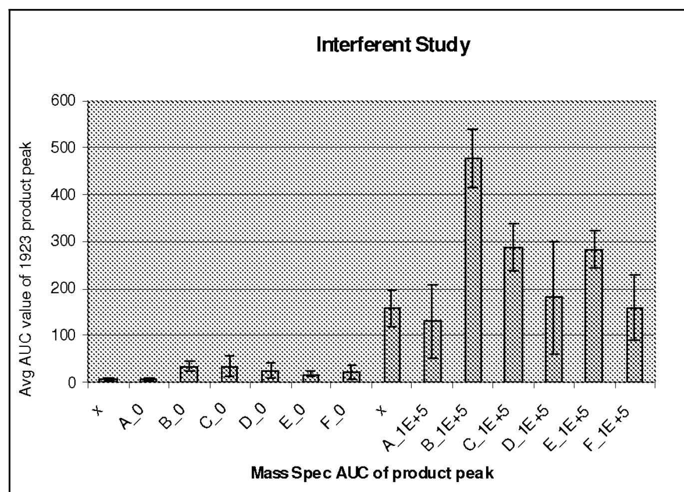

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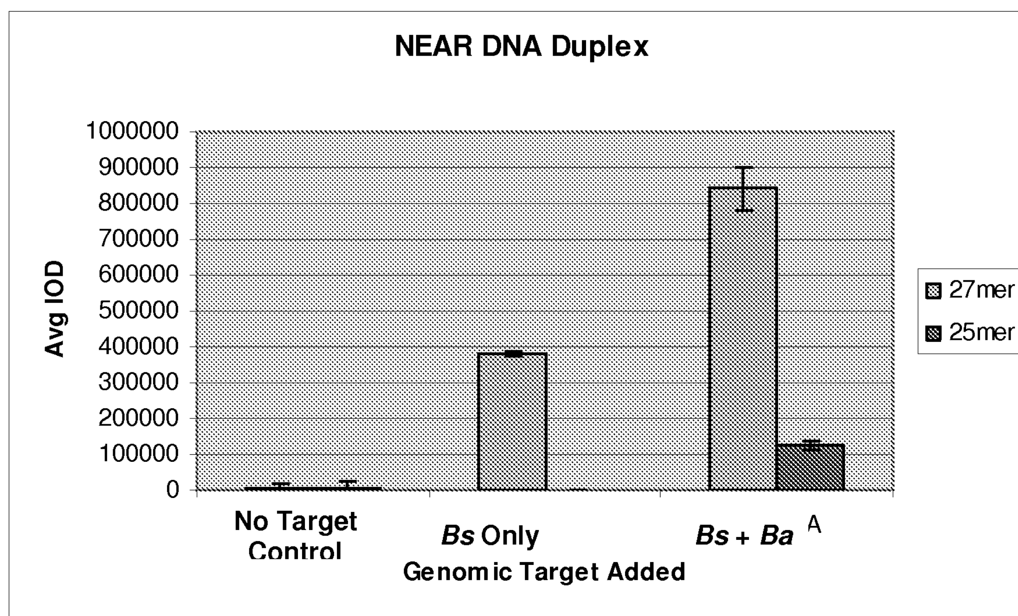

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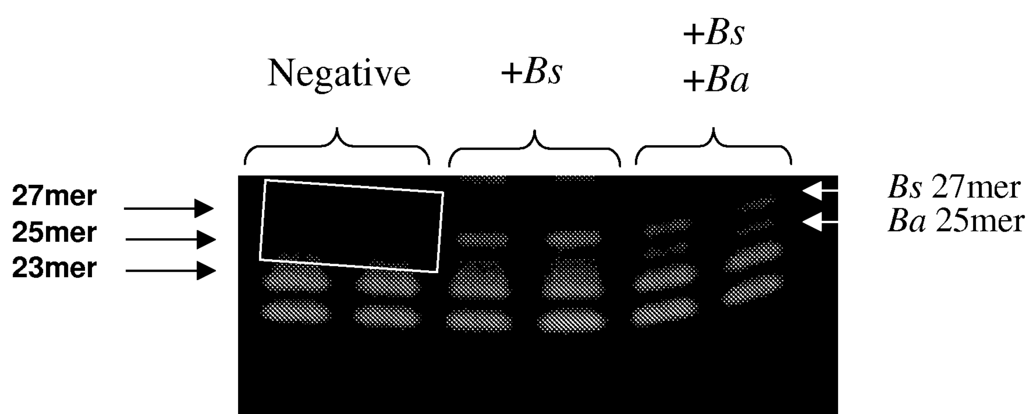

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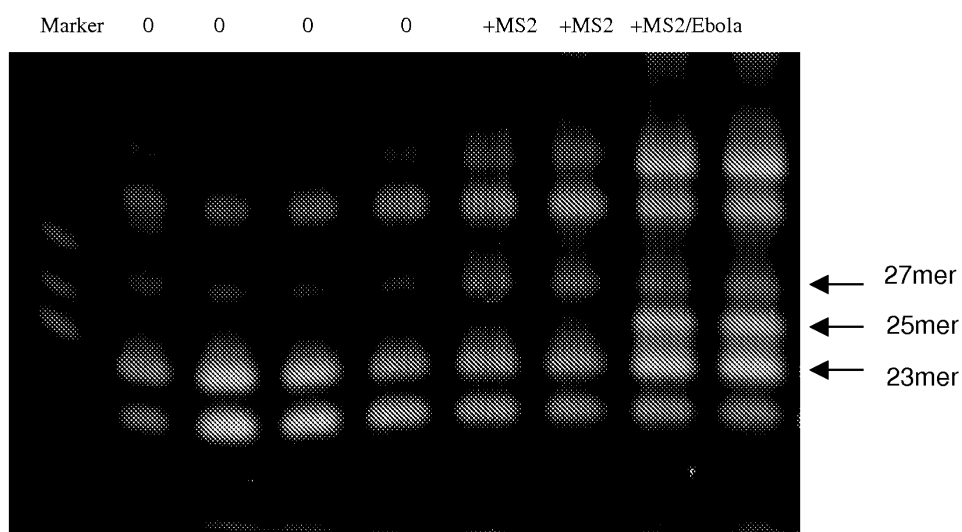

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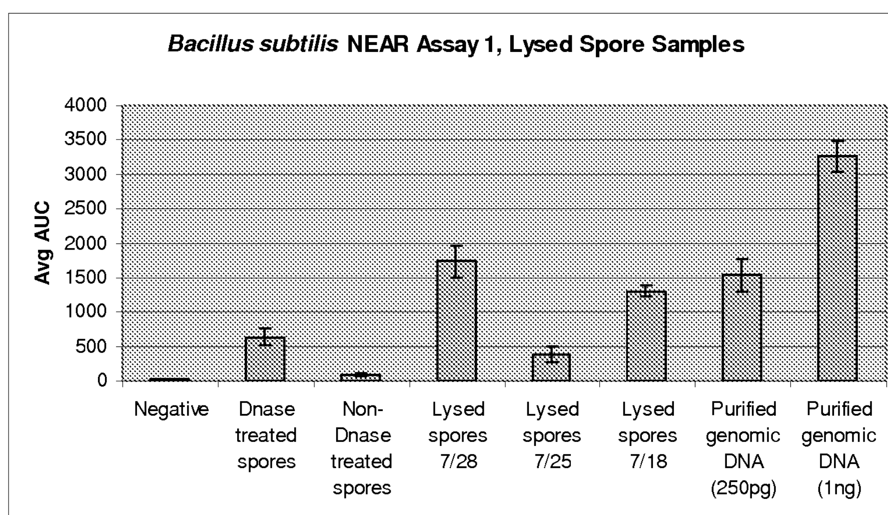

Figure 19

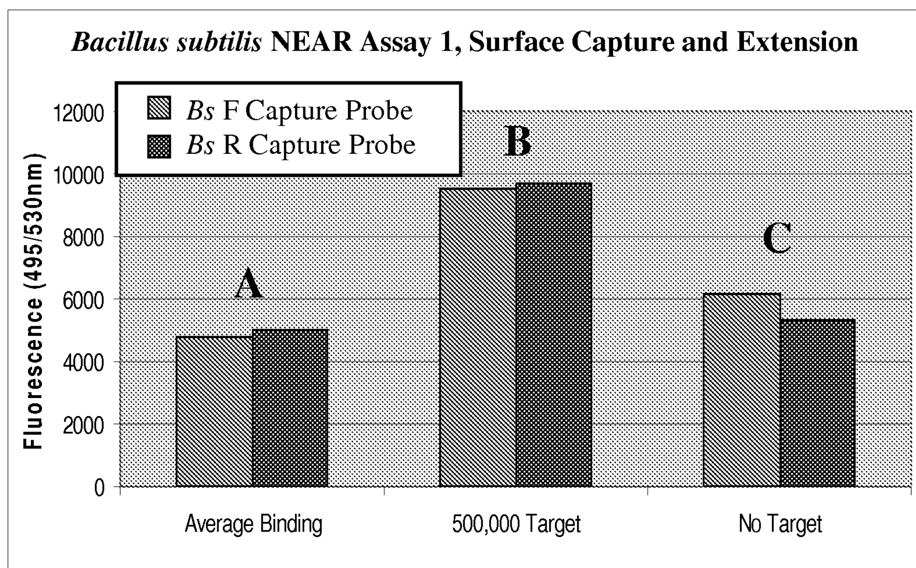

Figure 20

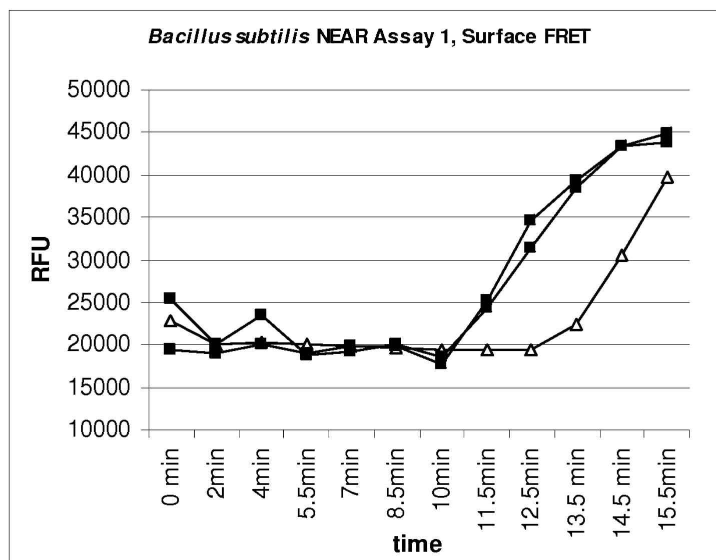

Figure 21

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DECLARATION (37 CFR 1.63) FOR UTILITY OR DESIGN APPLICATION USING AN APPLICATION DATA SHEET (37 CFR 1.76)

Title of Invention	NICKING AND EXTENSION AMPLIFICATION REACTION FOR THE EXPONENTIAL AMPLIFICATION OF NUCLEIC ACIDS

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[ ] United States application or PCT international application number ____________

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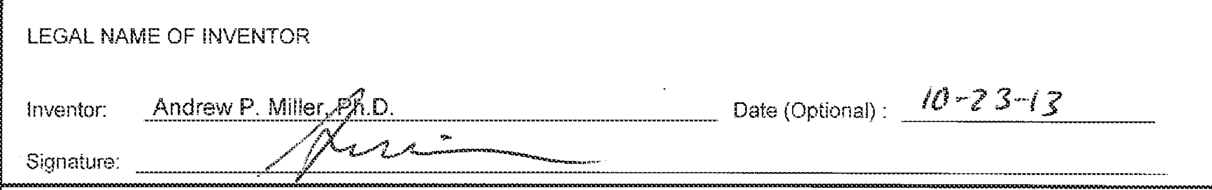
LEGAL NAME OF INVENTOR

Inventor: Andrew P. Miller, Ph.D. Date (Optional) : 10-23-13

Signature:

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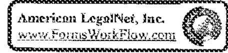

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LEGAL NAME OF INVENTOR

Inventor: Brian K. Maples Date (Optional) : ____________

Signature:

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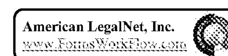

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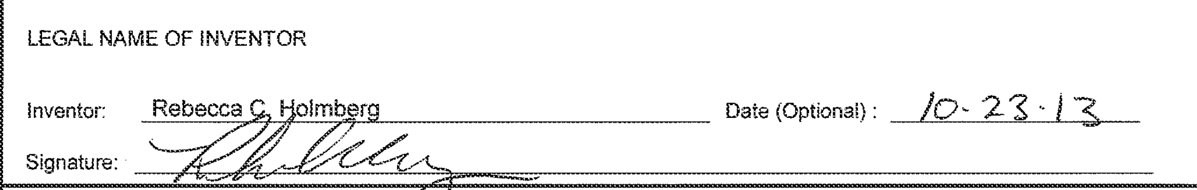
LEGAL NAME OF INVENTOR

Inventor: Rebecca C. Holmberg Date (Optional) : 10-23-13

Signature:



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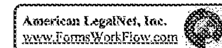

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LEGAL NAME OF INVENTOR

Inventor: Jarrod Provins   Date (Optional): 10/29/13

Signature: ______

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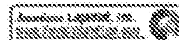

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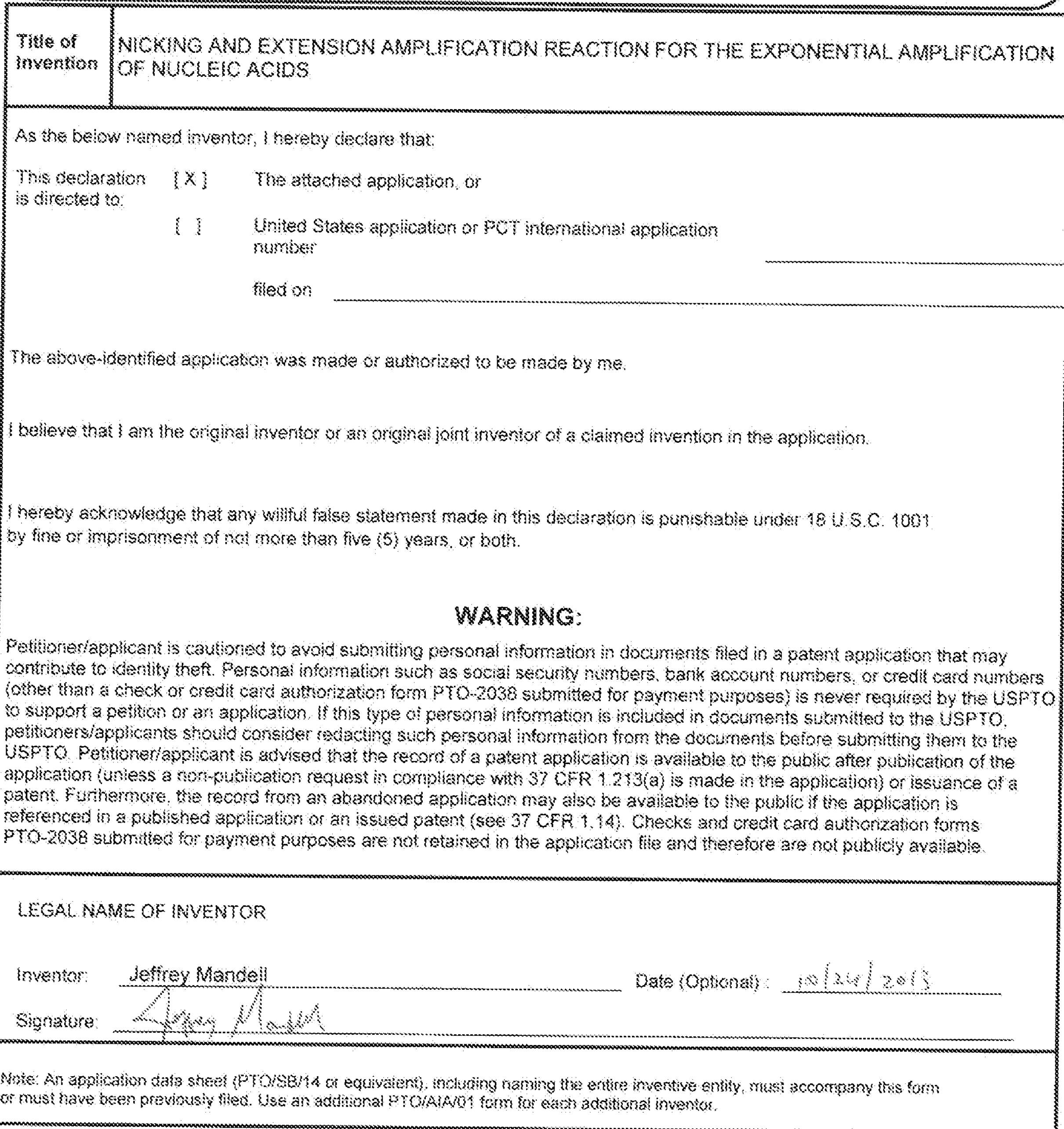

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LEGAL NAME OF INVENTOR

Inventor: Jeffrey Mandell Date (Optional): 10/24/2013

Signature: [signature]

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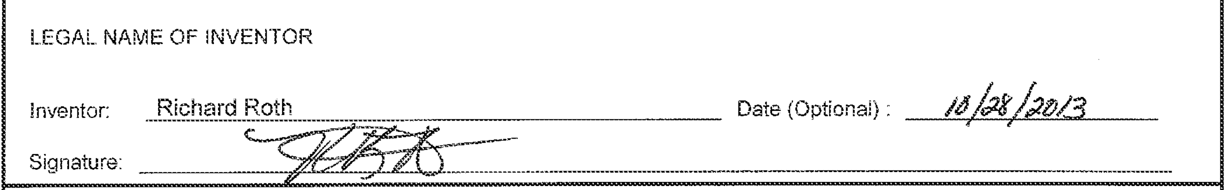

LEGAL NAME OF INVENTOR

Inventor: Richard Roth Date (Optional): 10/28/2013

Signature:

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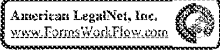

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