PTO/SB/05 (08-08)
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U.S. Patent and Trademark Office. U.S. DEPARTMENT OF COMMERCE

UTILITY PATENT APPLICATION TRANSMITTAL

(Only for new nonprovisional applications under 37 CFR 1.53(b))

Attorney Docket No.	001107.00866
First Inventor	Bert VOGELSTEIN et al.
Title	DIGITAL AMPLIFICATION
Express Mail Label No.	

APPLICATION ELEMENTS

See MPEP chapter 600 concerning utility patent application contents.

1. ☐ **Fee Transmittal Form** (e.g., PTO/SB/17)
2. ☐ **Applicant claims small entity status.** See 37 CFR 1.27.
3. ☑ **Specification** [*Total Pages* 30]
   Both the claims and abstract must start on a new page
   (For information on the preferred arrangement, see MPEP 608.01(a))
4. ☑ **Drawing(s)** (*35 U.S.C. 113*) [*Total Sheets* 7]
5. **Oath or Declaration** [*Total Sheets* 2]
   a. ☐ Newly executed (original or copy)
   b. ☑ A copy from a prior application (37 CFR 1.63(d))
   (for continuation/divisional with Box 18 completed)
   i. ☐ DELETION OF INVENTOR(S)
   Signed statement attached deleting inventor(s) name in the prior application, see 37 CFR 1.63(d)(2) and 1.33(b).
6. ☑ **Application Data Sheet.** See 37 CFR 1.76
7. ☐ **CD-ROM or CD-R** in duplicate, large table or Computer Program *(Appendix)*
   ☐ Landscape Table on CD
8. **Nucleotide and/or Amino Acid Sequence Submission**
   (if applicable, items a. – c. are required)
   a. ☐ Computer Readable Form (CRF)
   b. Specification Sequence Listing on:
      i. ☐ CD-ROM or CD-R (2 copies); or
      ii. ☑ Paper
   c. ☑ Statements verifying identity of above copies

ADDRESS TO: **Commissioner for Patents**
P.O. Box 1450
Alexandria VA 22313-1450

ACCOMPANYING APPLICATION PARTS

9. ☐ **Assignment Papers** (cover sheet & document(s))
   Name of Assignee The Johns Hopkins University
10. ☐ **37 CFR 3.73(b) Statement** *(when there is an assignee)* ☐ **Power of Attorney**
11. ☐ **English Translation Document** *(if applicable)*
12. ☑ **Information Disclosure Statement** (PTO/SB/08 or PTO-1449)
    ☐ Copies of citations attached
13. ☐ **Preliminary Amendment**
14. ☐ **Return Receipt Postcard** (MPEP 503)
    (*Should be specifically itemized)*
15. ☐ **Certified Copy of Priority Document(s)**
    (if foreign priority is claimed)
16. ☐ **Nonpublication Request** under 35 U.S.C. 122(b)(2)(B)(i). Applicant must attach form PTO/SB/35 or equivalent.
17. ☑ Other: Please use the CRF from parent 09/981,356 filed on Nov. 14, 2003. Contents are identical.

18. If a CONTINUING APPLICATION, *check appropriate box, and supply the requisite information below and in the first sentence of the specification following the title, or in an Application Data Sheet under 37 CFR 1.76:*

☑ Continuation ☐ Divisional ☐ Continuation-in-part (CIP) of prior application No.: 12/617,368

Prior application information: *Examiner* Samuel C. WOOLWINE *Art Unit:* 1637

19. CORRESPONDENCE ADDRESS

☑ The address associated with Customer Number: 22907 ***OR*** ☐ Correspondence address below

Name					
Address					
City		State		Zip Code	
Country		Telephone		Email	

Signature	/Sarah A. Kagan/		Date	March 24, 2011	
Name (Print/Type)	Sarah A. Kagan			Registration No. (Attorney/Agent)	32,141

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.

PTO/SB/14 (11-08)
Approved for use through 09/30/2010. OMB 0651-0032
U.S. Patent and Trademark Office; U.S. DEPARTMENT OF COMMERCE

Application Data Sheet 37 CFR 1.76	Attorney Docket Number	001107.00866
	Application Number	
Title of Invention	Digital Amplification	

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.)

Applicant Information:

Applicant 1 Remove

Applicant Authority ◉ Inventor ○ Legal Representative under 35 U.S.C. 117 ○ Party of Interest under 35 U.S.C. 118

Prefix	Given Name	Middle Name	Family Name	Suffix
	Bert		VOGELSTEIN	

Residence Information (Select One) ◉ US Residency ○ Non US Residency ○ Active US Military Service

City	Baltimore	State/Province	MD	Country of Residence i	US

Citizenship under 37 CFR 1.41(b) i: US

Mailing Address of Applicant:

Address 1	3700 Breton Way		
Address 2			
City	Baltimore	State/Province	MD
Postal Code	21208	Country i	US

Applicant 2 Remove

Applicant Authority ◉ Inventor ○ Legal Representative under 35 U.S.C. 117 ○ Party of Interest under 35 U.S.C. 118

Prefix	Given Name	Middle Name	Family Name	Suffix
	Kenneth	W.	KINZLER	

Residence Information (Select One) ◉ US Residency ○ Non US Residency ○ Active US Military Service

City	Baltimore	State/Province	MD	Country of Residence i	US

Citizenship under 37 CFR 1.41(b) i: US

Mailing Address of Applicant:

Address 1	1403 Halkirk Way		
Address 2			
City	Baltimore	State/Province	MD
Postal Code	21015	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.**

Application Data Sheet 37 CFR 1.76	Attorney Docket Number	001107.00866
	Application Number	
Title of Invention	Digital Amplification	

Customer Number	22907		
Email Address		Add Email	Remove Email

Application Information:

Title of the Invention	Digital Amplification		
Attorney Docket Number	001107.00866	Small Entity Status Claimed	☐
Application Type	Nonprovisional		
Subject Matter	Utility		
Suggested Class (if any)		Sub Class (if any)	
Suggested Technology Center (if any)			
Total Number of Drawing Sheets (if any)	7	Suggested Figure for Publication (if any)	1A

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).
Enter either 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	22907		

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(a)(2) or CFR 1.78(a)(4), and need not otherwise be made part of the specification.

Prior Application Status	Pending		Remove
Application Number	Continuity Type	Prior Application Number	Filing Date (YYYY-MM-DD)
	Continuation of	12617368	2009-11-12
Prior Application Status	Patented		Remove

Application Data Sheet 37 CFR 1.76	Attorney Docket Number	001107.00866
	Application Number	
Title of Invention	Digital Amplification	

Application Number	Continuity Type	Prior Application Number	Filing Date (YYYY-MM-DD)	Patent Number	Issue Date (YYYY-MM-DD)
12617368	Continuation of	11709742	2007-02-23	7824889	2010-11-02

Prior Application Status	Abandoned	Remove

Application Number	Continuity Type	Prior Application Number	Filing Date (YYYY-MM-DD)
11709742	Continuation of	10828295	2004-04-21

Prior Application Status	Patented	Remove

Application Number	Continuity Type	Prior Application Number	Filing Date (YYYY-MM-DD)	Patent Number	Issue Date (YYYY-MM-DD)
10828295	Division of	09981356	2001-10-12	6753147	2004-06-22

Prior Application Status	Patented	Remove

Application Number	Continuity Type	Prior Application Number	Filing Date (YYYY-MM-DD)	Patent Number	Issue Date (YYYY-MM-DD)
09981356	Continuation of	09613826	2000-07-11	6440706	2002-08-27

Prior Application Status	Expired	Remove

Application Number	Continuity Type	Prior Application Number	Filing Date (YYYY-MM-DD)
09613826	non provisional of	60146792	1999-08-02

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 benefit of foreign priority and to identify any prior foreign application for which priority is not claimed. 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(a).

Remove

Application Number	Country [i]	Parent Filing Date (YYYY-MM-DD)	Priority Claimed
			(•) Yes ( ) No

Additional Foreign Priority Data may be generated within this form by selecting the **Add** button. Add

Assignee Information:

Providing this information in the application data sheet does not substitute for compliance with any requirement of part 3 of Title 37 of the CFR to have an assignment recorded in the Office.

Assignee 1 Remove

If the Assignee is an Organization check here. [X]

Organization Name	The Johns Hopkins University

Application Data Sheet 37 CFR 1.76	Attorney Docket Number	001107.00866
	Application Number	
Title of Invention	Digital Amplification	

Mailing Address Information:			
Address 1	3400 N. Charles Street		
Address 2			
City	Baltimore	**State/Province**	MD
Country [i]		Postal Code	21218
Phone Number		Fax Number	
Email Address			
Additional Assignee Data may be generated within this form by selecting the **Add** button.		Add	

Signature:

A signature of the applicant or representative is required in accordance with 37 CFR 1.33 and 10.18. Please see 37 CFR 1.4(d) for the form of the signature.					
Signature	/Sarah A. Kagan/			Date (YYYY-MM-DD)	2011-03-24
First Name	Sarah A.	Last Name	Kagan	Registration Number	32141

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.

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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.

JOINT DECLARATION FOR PATENT APPLICATION

As the below named inventor, we hereby declare that:

Our residence, post office address and citizenship are as stated below next to our names;

We believe we are the original, first and joint inventors of the subject matter which is claimed and for which a patent is sought on the invention entitled DIGITAL AMPLIFICATION, the specification of which

- ☐ is attached hereto.
- ■ was filed on July 11, 2000 as Application Serial Number 09/613,826 and was amended on (if applicable).
- ☐ was filed under the Patent Cooperation Treaty (PCT) and accorded International Application No. _____, filed _____, and amended on _____ (if any).

We hereby state that we have reviewed and understand the contents of the above identified specification, including the claims, as amended by any amendment referred to above.

We hereby acknowledge the duty to disclose information which is material to patentability in accordance with Title 37, Code of Federal Regulations, §1.56(a).

Prior Foreign Application(s)

We hereby claim foreign priority benefits under Title 35, United States Code, §119 of any foreign application(s) for patent or inventor's certificate listed below and have also identified below any foreign application(s) for patent or inventor's certificate having a filing date before that of the application on which priority is claimed:

Country	Application No.	Date of Filing (day month year)	Date of Issue (day month year)	Priority Claimed Under 35 U.S.C. §119

Prior United States Provisional Application(s)

We hereby claim priority benefits under Title 35, United States Code, §119(e)(1) of any U.S. provisional application listed below:

U.S. Provisional Application No.	Date of Filing (day month year)	Priority Claimed Under 35 U.S.C. §119(e)(1)
60/146,792	02 August 1999	Yes

Prior United States Application(s)

We hereby claim the benefit under Title 35, United States Code, §120 of any United States application(s) listed below and, insofar as the subject matter of each of the claims of this application is not disclosed in the prior United States application in the manner provided by the first paragraph of Title 35, United States Code, §112, we acknowledge the duty to disclose material information as defined in Title 37, Code of Federal Regulations, §1.56(a) which occurred between the filing date of the prior application and the national or PCT international filing date of this application:

Application Serial No.	Date of Filing (Day, Month, Year)	Status — Patented, Pending, Abandoned

Power of Attorney

And we hereby appoint, both jointly and severally, as our attorneys with full power of substitution and revocation, to prosecute this application and to transact all business in the Patent and Trademark Office connected herewith the following attorneys and agents, their registration numbers being listed after their names:

ALTHERR, Robert F.	31,810	HOSCHEIT, Dale H.	19,090	PATEL, Binal J.	42,065
BANNER, Donald W.	17,037	IWANICKI, John P.	34,628	PATHAK, Ajay S.	38,266
BANNER, Mark T.	29,888	JACKSON, Thomas H.	29,808	PAYNE, Stephen S.	35,316
BANNER, Pamela I.	33,644	KAGAN, Sarah A.	32,141	PETERSON, Thomas L.	30,969
BECKETT, William W.	18,262	KATZ, Robert S.	36,402	POTENZA, Joseph M.	28,175
BODNER, Jordan	42,338	KLEIN, William J.	43,719	PRATT, Thomas K.	37,210
BUROW, Scott A.	42,373	KRAUSE, Joseph P.	32,578	RENK, Christopher J.	33,761
CALLAHAN, James V.	20,095	LINEK, Ernest V.	29,822	RESIS, Robert H.	32,168
CHANG, Steve S	42,402	MALONE, Dale A.	32,155	RIVARD, Paul M.	43,446
COHAN, Gregory J.	40,959	MANNAVA, Ashok K.	45,301	SCHAD, Steve P.	32,550
COOPERMAN, Marc S.	34,143	McDERMOTT, Peter D.	29,411	SHANAHAN, Michael H.	24,438
CURTIN, Joseph P.	34,571	McKEE, Christopher L.	32,384	SHIFLEY, Charles W.	28,042
DAWSON, John R.	39,504	McKIE, Edward F.	17,335	SKERPON, Joseph M.	29,864
DEMOOR, Laura J.	39,654	MEDLOCK, Nina L.	29,673	STOCKLEY, D. J.	34,257
EVANS, Thomas L.	35,805	MEECE, Timothy C.	38,553	VAN ES, J. Pieter	37,746
FEDOROCHKO, Gary D.	35,509	MEEKER, Frederic M.	35,282	WITCOFF, Sheldon W.	17,399
FISHER, William J.	32,133	MILLER, Charles L.	43,805	WOLFFE, Franklin D.	19,724
GLEMBOCKI, Christopher	R.38,800	MITRIUS, Janice V.	43,808	WOLFFE, Susan A.	33,568
HANLON, Brian E.	40,449	MORENO, Christopher P.	38,566	WRIGHT, Bradley C.	38,061
HEMMENDINGER, Lisa M.	42,653	NELSON, Jon O.	24,566		
HONG, Patricia E.	34,373	NIEGOWSKI, James A.	28,331		

All correspondence and telephone communications should be addressed to:

Banner & Witcoff, Ltd.
1001 G Street, N.W., 11th Floor
Washington, D.C. 20001-4597

Customer Number: 22907
Tel: (202) 508-9100
Fax: (202) 508-9299

We hereby declare that all statements made herein of our 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 issuing thereon.

Signature _____ Date 11/28/00

Full Name of First Inventor: Vogelstein (Family Name) Bert (First Given Name) _____ (Second Given Name)

Residence Baltimore, Maryland Citizenship United States

Post Office Address 3700 Breton Way, Baltimore, Maryland 21208

Signature _____ Date 11/28/00

Full Name of Second Inventor: Kinzler (Family Name) Kenneth (First Given Name) W. (Second Given Name)

Residence BelAir, Maryland Citizenship United States

Post Office Address 1403 Halkirk Way, BelAir, Maryland 21015

PATENT

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re Application of	)	Prior Group Art Unit: 1637
	)	
Bert VOGELSTEIN et al	)	Prior Examiner: Samuel Woolwine
	)	
Continuation Application of	)	Confirmation No. TBD
Serial No. 12/617,368	)	
	)	Atty. Dkt. No. 001107.00866
Filed: Herewith	)	
	)	
For: DIGITAL AMPLIFICATION	)	

RECOGNITION OF PRACTITIONERS OF RECORD UNDER 37 C.F.R. § 1.32(c)(3)

U.S. Patent and Trademark Office
Customer Service Window
Randolph Building
401 Dulany Street
Alexandria, VA 22314

Sir:

Pursuant to 37 C.F.R. § 1.32(c)(3), please recognize the following patent practitioners, originally named in the Power of Attorney from an earlier-filed application, as being of record in the above-identified application:

Name	Registration No.
Sarah A. Kagan	32,141
Dale H. Hoscheit	19,090
Joseph M. Skerpon	29,864
Lisa M. Hemmendinger	42,653
William J. Fisher	32,133

A copy of the Power of Attorney from the earlier-filed application is submitted herewith.

Respectfully submitted,
BANNER & WITCOFF, LTD.

Dated: March 24, 2011

By: */SARAH A. KAGAN/*
Sarah A. Kagan
Registration No. 32,141

Customer No. 22907

DIGITAL AMPLIFICATION

The U.S. government retains certain rights in this invention by virtue of its support of the underlying research, supported by grants CA 43460, CA 57345, and CA 62924 from the National Institutes of Health.

The disclosure of all claimed priority applications is expressly incorporated herein.

TECHNICAL FIELD OF THE INVENTION

This invention is related to diagnostic genetic analyses. In particular it relates to detection of genetic changes and gene expression.

BACKGROUND OF THE INVENTION

In classical genetics, only mutations of the germ-line were considered important for understanding disease. With the realization that somatic mutations are the primary cause of cancer, and may also play a role in aging, new genetic principles have arisen. These discoveries have provided a wealth of new opportunities for patient management as well as for basic research into the pathogenesis of neoplasia. However, many of these opportunities hinge upon detection of a small number of mutant-containing cells among a large excess of normal cells. Examples include the detection of neoplastic cells in urine, stool, and sputum of patients with cancers of the bladder, colorectum, and lung, respectively. Such detection has been shown in some cases to be possible at a stage when the primary tumors are still curable and the patients asymptomatic. Mutant sequences from the DNA of neoplastic cells have also been found in the blood of cancer patients. The detection of residual disease in lymph nodes or surgical margins may be useful in predicting which patients

might benefit most from further therapy. From a basic research standpoint, analysis of the early effects of carcinogens is often dependent on the ability to detect small populations of mutant cells.

Because of the importance of this issue in so many settings, many useful techniques have been developed for the detection of mutations. DNA sequencing is the gold standard for the detection of germ line mutations, but is useful only when the fraction of mutated alleles is greater than ~20%. Mutant-specific oligonucleotides can sometimes be used to detect mutations present in a minor proportion of the cells analyzed, but the signal to noise ratio distinguishing mutant and wild-type (WT) templates is variable. The use of mutant-specific primers or the digestion of polymerase chain reaction (PCR) products with specific restriction endonucleases are extremely sensitive methods for detecting such mutations, but it is difficult to quantitate the fraction of mutant molecules in the starting population with these techniques. Other innovative approaches for the detection of somatic mutations have been reviewed. A general problem with these methods is that it is difficult or impossible to independently confirm the existence of any mutations that are identified.

Thus there is a need in the art for methods for accurately and quantitatively detecting genetic sequences in mixed populations of sequences.

SUMMARY OF THE INVENTION

It is an object of the present invention to provide methods for determining the presence of a selected genetic sequence in a population of genetic sequences.

It is another object of the present invention to provide molecular beacon probes useful in the method of the invention.

These and other objects of the invention are achieved by providing a method for determining the presence of a selected genetic sequence in a

population of genetic sequences. A biological sample comprising nucleic acid template molecules is diluted to form a set of assay samples. The template molecules within the assay samples are amplified to form a population of amplified molecules in the assay samples of the set. The amplified molecules in the assay samples of the set are then analyzed to determine a first number of assay samples which contain the selected genetic sequence and a second number of assay samples which contain a reference genetic sequence. The first number is then compared to the second number to ascertain a ratio which reflects the composition of the biological sample.

Another embodiment of the invention is a method for determining the ratio of a selected genetic sequence in a population of genetic sequences. Template molecules within a set comprising a plurality of assay samples are amplified to form a population of amplified molecules in each of the assay samples of the set. The amplified molecules in the assay samples of the set are analyzed to determine a first number of assay samples which contain the selected genetic sequence and a second number of assay samples which contain a reference genetic sequence. At least one-fiftieth of the assay samples in the set comprise a number (N) of molecules such that 1/N is larger than the ratio of selected genetic sequences to total genetic sequences required to determine the presence of the selected genetic sequence. The first number is compared to the second number to ascertain a ratio which reflects the composition of the biological sample.

According to another embodiment of the invention, a molecular beacon probe is provided. It comprises an oligonucleotide with a stem-loop structure having a photoluminescent dye at one of the 5' or 3' ends and a quenching agent at the opposite 5' or 3' end. The loop consists of 16 base pairs which has a T_m of 50-51 C. The stem consists of 4 base pairs having a sequence 5'-CACG-3'.

A second type of molecular beacon probe is provided in another embodiment. It comprises an oligonucleotide with a stem-loop structure having a photoluminescent dye at one of the 5' or 3' ends and a quenching agent at the opposite 5' or 3' end. The loop consists of 19-20 base pairs and has a T_m of 54-56 C. The stem consists of 4 base pairs having a sequence 5'-CACG-3'.

Another embodiment provides the two types of molecular beacon probes, either mixed together or provided in a divided container as a kit.

The invention thus provides the art with the means to obtain quantitative assessments of particular DNA or RNA sequences in mixed populations of sequences using digital (binary) signals.

BRIEF DESCRIPTION OF THE DRAWINGS

FIG. 1A, 1B, 1C. Schematic of experimental design. (Fig. 1A) The basic two steps involved: PCR on diluted DNA samples is followed by addition of fluorescent probes which discriminate between WT and mutant alleles and subsequent fluorometry. (Fig. 1B) Principle of molecular beacon analysis. In the stem-loop configuration, fluorescence from a dye at the 5' end of the oligonucleotide probe is quenched by a Dabcyl group at the 3' end. Upon hybridization to a template, the dye is separated from the quencher, resulting in increased fluorescence. Modified from Marras *et al.* (Fig. 1C) Oligonucleotide design. Primers F1 and R1 are used to amplify the genomic region of interest. Primer INT is used to produce single stranded DNA from the original PCR products during a subsequent asymmetric PCR step (see Materials and Methods). MB-RED is a Molecular Beacon which detects any appropriate PCR product, whether it is WT or mutant at the queried codons. MB-GREEN is a Molecular Beacon which preferentially detects the WT PCR product.

FIG. 2. Discrimination between WT and mutant PCR products by Molecular Beacons. Ten separate PCR products, each generated from ~25 genome equivalents of genomic DNA of cells containing the indicated mutations of *c-Ki-Ras*, were analyzed with the Molecular Beacon probes described in the text. Representative examples of the PCR products used for Molecular Beacon analysis were purified and directly sequenced. In the cases with Gly12Cys (SEQ ID NO: 11) and Gly12Arg (SEQ ID NO: 10) mutations, contaminating non-neoplastic cells within the tumor presumably accounted for the relatively low ratios. In the cases with Gly12Ser (SEQ ID NO: 8) and Gly12Asp (SEQ ID NO: 12), there were apparently two or more alleles of mutant *c-Ki-Ras* for every WT allele (SEQ ID NO: 7); both these tumors were aneuploid. Analysis of the Gly13Asp mutation is also shown (SEQ ID NO: 9).

FIG. 3. Detecting Dig-PCR products with MB-RED. Specific Fluorescence Units of representative wells from an experiment employing colorectal cancer cells with Gly12Asp or Gly13Asp mutations of the *c-Ki-Ras* gene. Wells with values >10,000 are shaded yellow. Polyacrylamide gel electrophoretic analyses of the PCR products from selected wells are shown. Wells with fluorescence values <3500 had no PCR product of the correct size while wells with fluorescence values >10,000 SFU always contained PCR products of 129 bp. Non-specific products generated during the large number of cycles required for Dig-PCR did not affect the fluorescence analysis. M1 and M2 are molecular weight markers used to determine the size of fragments indicated on the left (in base pairs).

FIG. 4. Discriminating WT from mutant PCR products obtained in Dig-PCR. RED/GREEN ratios were determined from the fluorescence of MB-RED and MB-GREEN as described in Materials and Methods. The wells shown are the same as those illustrated in Fig. 3. The sequences of PCR products from the

indicated wells were determined as described in Materials and Methods. The wells with RED/GREEN ratios >3.0 each contained mutant sequences while those with RED/GREEN ratios of ~1.0 contained WT sequences. WT c-Ki-Ras (SEQ ID NO: 7), Gly12Asp (SEQ ID NO: 13), and Gly13Asp (SEQ ID NO: 9) were analyzed.

FIG. 5. Dig-PCR of DNA from a stool sample. The 384 wells used in the experiment are displayed. Those colored blue contained 25 genome equivalents of DNA from normal cells. Each of these registered positive with MB-RED and the RED/GREEN ratios were 1.0 +/- 0.1 (mean +/- 1 standard deviation). The wells colored yellow contained no template DNA and each was negative with MB-RED (i.e., fluorescence <3500 fluorescence units.). The other wells contained diluted DNA from the stool sample. Those registering as positive with MB-RED were colored either red or green, depending on their RED/GREEN ratios. Those registering negative with MB-RED were colored white. PCR products from the indicated wells were used for automated sequence analysis. The sequence of WT *c-Ki-Ras* in well (SEQ ID NO: 7), and mutant *c-Ki-Ras* in wells C10, E11, M10, and L12 (SEQ ID NO: 14), and well F21 (SEQ ID NO: 15) were analyzed.

DETAILED DESCRIPTION OF THE INVENTION

The method devised by the present inventors involves separately amplifying small numbers of template molecules so that the resultant products have a proportion of the analyte sequence which is detectable by the detection means chosen. At its limit, single template molecules can be amplified so that the products are completely mutant or completely wild-type (WT). The homogeneity of these amplification products makes them trivial to distinguish through existing techniques.

The method requires analyzing a large number of amplified products simply and reliably. Techniques for such assessments were developed, with the output providing a digital readout of the fraction of mutant alleles in the analyzed population.

The biological sample is diluted to a point at which a practically usable number of the diluted samples contain a proportion of the selected genetic sequence (analyte) relative to total template molecules such that the analyzing technique being used can detect the analyte. A practically usable number of diluted samples will depend on cost of the analysis method. Typically it would be desirable that at least 1/50 of the diluted samples have a detectable proportion of analyte. At least 1/10, 1/5, 3/10, 2/5, 1/2, 3/5, 7/10, 4/5, or 9/10 of the diluted samples may have a detectable proportion of analyte. The higher the fraction of samples which will provide useful information, the more economical will be the overall assay. Over-dilution will also lead to a loss of economy, as many samples will be analyzed and provide no signal. A particularly preferred degree of dilution is to a point where each of the assay samples has on average one-half of a template. The dilution can be performed from more concentrated samples. Alternatively, dilute sources of template nucleic acids can be used. All of the samples may contain amplifiable template molecules. Desirably each assay sample prior to amplification will contain less than a hundred or less than ten template molecules.

Digital amplification can be used to detect mutations present at relatively low levels in the samples to be analyzed. The limit of detection is defined by the number of wells that can be analyzed and the intrinsic mutation rate of the polymerase used for amplification. 384 well PCR plates are commercially available and 1536 well plates are on the horizon, theoretically allowing sensitivities for mutation detection at the ~0.1% level. It is also possible that Digital Amplification can be performed in microarray format, potentially increasing the sensitivity by another order of magnitude. This

sensitivity may ultimately be limited by polymerase errors. The effective error rate in PCR as performed under our conditions was 1.1%, i.e., four out of 351 PCR products derived from WT DNA sequence appeared to contain a mutation by RED/GREEN ratio criteria. However, any individual mutation (such as a G to T transversion at the second position of codon 12 of *c-Ki-Ras*), are expected to occur in < 1 in 50 of these polymerase-generated mutants (there are at least 50 base substitutions within or surrounding codons 12 and 13 that should yield high RED/GREEN ratios). Determining the sequence of the putative mutants in the positive wells, by direct sequencing as performed here or by any of the other techniques, provides unequivocal validation of a prospective mutation: a significant fraction of the mutations found in individual wells should be identical if the mutation occurred *in vivo*. Significance can be established through rigorous statistical analysis, as positive signals should be distributed according to Poisson probabilities. Moreover, the error rate in particular Digital Amplification experiments can be precisely determined through performance of Digital Amplification on DNA templates from normal cells.

Digital Amplification is as easily applied to RT-PCR products generated from RNA templates as it is to genomic DNA. For example, the fraction of alternatively spliced or mutant transcripts from a gene can be easily determined using photoluminescent probes specific for each of the PCR products generated. Similarly, Digital Amplification can be used to quantitate relative levels of gene expression within an RNA population. For this amplification, each well would contain primers which are used to amplify a reference transcript expressed constitutively as well as primers specific for the experimental transcript. One photoluminescent probe would then be used to detect PCR products from the reference transcript and a second photoluminescent probe used for the test transcript. The number of wells in which the test transcript is amplified divided by the number of wells in which

the reference transcript is amplified provides a quantitative measure of gene expression. Another group of examples involves the investigations of allelic status when two mutations are observed upon sequence analysis of a standard DNA sample. To distinguish whether one variant is present in each allele (*vs.* both occurring in one allele), cloning of PCR products is generally performed. The approach described here would simplify the analysis by eliminating the need for cloning. Other potential applications of Digital Amplification are listed in Table 1. When the goal is the quantitation of the proportion of two relatively common alleles or transcripts rather than the detection of rare alleles, techniques such as those employing TaqMan and real time PCR provide an excellent alternative to use of molecular beacons. Advantages of real time PCR methods include their simplicity and the ability to analyze multiple samples simultaneously. However, Digital Amplification may prove useful for these applications when the expected differences are small, (*e.g.*, only ~2-fold, such as occurs with allelic imbalances.)

Application	Example	Probe 1 Detects:	Probe 2 Detects:
Base substitution mutations	Cancer gene mutations in stool, blood, lymph nodes	mutant or WT alleles	WT PCR products
Chromosomal translocations	Residual leukemia cells after therapy (DNA or RNA)	normal or translocated alleles	translocated allele
Gene amplifications	Determine presence or extent of amplification	sequence within amplicon	sequence from another part of same chromosome arm
Alternatively spliced products	Determine fraction of alternatively spliced transcripts from same gene (RNA)	minor exons	common exons
Changes in gene expression	Determine relative levels of expression of two genes (RNA)	first transcript	reference transcript
Allelic discrimination	Two different alleles mutated vs. one mutation in each of two alleles	first mutation	second mutation
Allelic Imbalance	Quantitative analysis with non-polymorphic markers	marker sequence	marker from another chromosome

The ultimate utility of Digital Amplification lies in its ability to convert the intrinsically exponential nature of PCR to a linear one. It should thereby prove useful for experiments requiring the investigation of individual alleles, rare variants/mutations, or quantitative analysis of PCR products.

In one preferred embodiment each diluted sample has on average one half a template molecule. This is the same as one half of the diluted samples having one template molecule. This can be empirically determined by amplification. Either the analyte (selected genetic sequence) or the reference genetic sequence can be used for this determination. If the analysis method being used can detect analyte when present at a level of 20%, then one must dilute such that a significant number of diluted assay samples contain more than 20% of analyte. If the analysis method being used requires 100% analyte to detect, then dilution down to the single template molecule level will be required.

To achieve a dilution to approximately a single template molecule level, one can dilute such that between 0.1 and 0.9 of the assay samples yield an amplification product. More preferably the dilution will be to between 0.1 and 0.6, more preferably to between 0.3 and 0.5 of the assay samples yielding an amplification product.

The digital amplification method requires analysis of a large number of samples to get meaningful results. Preferably at least ten diluted assay samples are amplified and analyzed. More preferably at least 15, 20, 25, 30, 40, 50, 75, 100, 500, or 1000 diluted assay samples are amplified and analyzed. As in any method, the accuracy of the determination will improve as the number of samples increases, up to a point. Because a large number of samples must be analyzed, it is desirable to reduce the manipulative steps, especially sample transfer steps. Thus it is preferred that the steps of amplifying and analyzing are performed in the same receptacle. This makes the method an *in situ,* or "one-pot" method.

The number of different situations in which the digital amplification method will find application is large. Some of these are listed in Table 1. As shown in the examples, the method can be used to find a tumor mutation in a population of cells which is not purely tumor cells. As described in the

examples, a probe for a particular mutation need not be used, but diminution in binding to a wild-type probe can be used as an indicator of the presence of one or more mutations. Chromosomal translocations which are characteristic of leukemias or lymphomas can be detected as a measure of the efficacy of therapy. Gene amplifications are characteristic of certain disease states. These can be measured using digital amplification. Alternatively spliced forms of a transcript can be detected and quantitated relative to other forms of the transcript using digital amplification on cDNA made from mRNA. Similarly, using cDNA made from mRNA one can determine relative levels of transcription of two different genes. One can use digital amplification to distinguish between a situation where one allele carries two mutations and one mutation is carried on each of two alleles in an individual. Allelic imbalances often result from a disease state. These can be detected using digital amplification.

Biological samples which can be used as the starting material for the analyses may be from any tissue or body sample from which DNA or mRNA can be isolated. Preferred sources include stool, blood, and lymph nodes. Preferably the biological sample is a cell-free lysate.

Molecular beacon probes according to the present invention can utilize any photoluminescent moiety as a detectable moiety. Typically these are dyes. Often these are fluorescent dyes. Photoluminescence is any process in which a material is excited by radiation such as light, is raised to an excited electronic or vibronic state, and subsequently re-emits that excitation energy as a photon of light. Such processes include fluorescence, which denotes emission accompanying descent from an excited state with paired electrons (a "singlet" state) or unpaired electrons (a "triplet" state) to a lower state with the same multiplicity, *i.e.,* a quantum-mechanically "allowed" transition. Photoluminescence also includes phosphorescence which denotes emission accompanying descent from an excited triplet or singlet state to a lower state of

different multiplicity, *i.e.,* a quantum mechanically "forbidden" transition. Compared to "allowed" transitions, "forbidden" transitions are associated with relatively longer excited state lifetimes.

The quenching of photoluminescence may be analyzed by a variety of methods which vary primarily in terms of signal transduction. Quenching may be transduced as changes in the intensity of photoluminescence or as changes in the ratio of photoluminescence intensities at two different wavelengths, or as changes in photoluminescence lifetimes, or even as changes in the polarization (anisotropy) of photoluminescence. Skilled practitioners will recognize that instrumentation for the measurement of these varied photoluminescent responses are known. The particular ratiometric methods for the analysis of quenching in the instant examples should not be construed as limiting the invention to any particular form of signal transduction. Ratiometric measurements of photoluminescence intensity can include the measurement of changes in intensity, photoluminescence lifetimes, or even polarization (anisotropy).

Although the working examples demonstrate the use of molecular beacon probes as the means of analysis of the amplified dilution samples, other techniques can be used as well. These include sequencing, gel electrophoresis, hybridization with other types of probes, including TaqMan™ (dual-labeled fluorogenic) probes (Perkin Elmer Corp./Applied Biosystems, Foster City, Calif), pyrene-labeled probes, and other biochemical assays.

The above disclosure generally describes the present invention. A more complete understanding can be obtained by reference to the following specific examples which are provided herein for purposes of illustration only, and are not intended to limit the scope of the invention.

EXAMPLE 1

Step 1: PCR amplifications. The optimal conditions for PCR described in this section were determined by varying the parameters described in the Results. PCR was performed in 7 ul volumes in 96 well polypropylene PCR plates (RPI). The composition of the reactions was: 67 mM Tris, pH 8.8, 16.6 mM NH_4SO_4, 6.7 mM $MgCl_2$, 10 mM β-mercaptoethanol, 1 mM dATP, 1 mM dCTP, 1 mM dGTP, 1 mM TTP, 6% DMSO, 1 uM primer F1, 1 uM primer R1, 0.05 units/ul Platinum Taq polymerase (Life Technologies, Inc.), and "one-half genome equivalent" of DNA. To determine the amount of DNA corresponding to one-half genome equivalent, DNA samples were serially diluted and tested via PCR. The amount that yielded amplification products in half the wells, usually ~1 pg of total DNA, was defined as "one-half genome equivalent" and used in each well of subsequent Digital Amplification experiments. Fifty ul light mineral oil (Sigma M-3516) was added to each well and reactions performed in a HybAid Thermal cycler at the following temperatures: denaturation at 94° for one min; 60 cycles of 94° for 15 sec, 55° for 15 sec., 70° for 15 seconds; 70° for five minutes. Reactions were read immediately or stored at room temperature for up to 36 hours before fluorescence analysis.

EXAMPLE 2

Step 2: Fluorescence analysis. 3.5 ul of a solution with the following composition was added to each well: 67 mM Tris, pH 8.8, 16.6 mM NH_4SO_4, 6.7 mM $MgCl_2$, 10 mM β-mercaptoethanol, 1 mM dATP, 1 mM dCTP, 1 mM dGTP, 1 mM TTP, 6% DMSO, 5 uM primer INT, 1 uM MB-GREEN, 1 uM MB-RED, 0.1 units/ul Platinum Taq polymerase. The plates were centrifuged for 20 seconds at 6000 g and fluorescence read at excitation/emission wavelengths of 485 nm/530 nm for MB-GREEN and 530 nm/590 nm for MB-RED. This fluorescence in wells without template was typically 10,000 to

20,000 fluorescence "units", with about 75% emanating from the fluorometer background and the remainder from the MB probes. The plates were then placed in a thermal cycler for asymmetric amplification at the following temperatures: 94° for one minute; 10 - 15 cycles of 94° for 15 sec, 55° for 15 sec., 70° for 15 seconds; 60° for five minutes. The plates were then incubated at room temperature for at least 20 minutes and fluorescence measured as described above. The fluorescence readings obtained were stable for several hours. Specific fluorescence was defined as the difference in fluorescence before and after the asymmetric amplification. RED/GREEN ratios were defined as the specific fluorescence of MB-RED divided by that of MB-GREEN. RED/GREEN ratios were normalized to the ratio exhibited by the positive controls (25 genome equivalents of DNA from normal cells, as defined in Materials and Methods). We found that the ability of MB probes to discriminate between WT and mutant sequences under our conditions could not be reliably determined from experiments in which they were tested by hybridization to relatively short complementary single stranded oligonucleotides, and that actual PCR products had to be used for validation.

EXAMPLE 3

Oligonucleotides and DNA sequencing. Primer F1: 5'-CATGTTCTAATATAGTCACATTTTCA-3' (SEQ ID NO: 1); Primer R1: 5'-TCTGAATTAGCTGTATCGTCAAGG-3' (SEQ ID NO: 2); Primer INT: 5'-TAGCTGTATCGTCAAGGCAC-3' (SEQ ID NO: 3); MB-RED: 5'-Cy3-CACGGGCCTGCTGAAAATGACTGCGTG-Dabcyl-3' (SEQ ID NO: 4); MB-GREEN: 5'-Fluorescein-CACGGGAGCTGGTGGCGTAGCGTG-Dabcyl-3'. (SEQ ID NO: 5).

Molecular Beacons were synthesized by Midland Scientific and other oligonucleotides were synthesized by Gene Link. All were dissolved at 50 uM

in TE (10 mM Tris, pH 8.0/ 1 mM EDTA) and kept frozen and in the dark until use. PCR products were purified using QIAquick PCR purification kits (Qiagen). In the relevant experiments described in the text, 20% of the product from single wells was used for gel electrophoresis and 40% was used for each sequencing reaction. The primer used for sequencing was 5'-CATTATTTTTATTATAAGGCCTGC-3' (SEQ ID NO: 6). Sequencing was performed using fluorescently-labeled ABI Big Dye terminators and an ABI 377 automated sequencer.

EXAMPLE 4

Principles underlying experiment. The experiment is outlined in Fig. 1A. First, the DNA is diluted into multiwell plates so that there is, on average, one template molecule per two wells, and PCR is performed. Second, the individual wells are analyzed for the presence of PCR products of mutant and WT sequence using fluorescent probes.

As the PCR products resulting from the amplification of single template molecules should be homogeneous in sequence, a variety of standard techniques could be used to assess their presence. Fluorescent probe-based technologies, which can be performed on the PCR products "*in situ*" (i.e., in the same wells) are particularly well-suited for this application. We chose to explore the utility of one such technology, involving Molecular Beacons (MB), for this purpose. MB probes are oligonucleotides with stem-loop structures that contain a fluorescent dye at the 5' end and a quenching agent (Dabcyl) at the 3' end (Fig. 1B). The degree of quenching via fluorescence energy resonance transfer is inversely proportional to the 6^{th} power of the distance between the Dabcyl group and the fluorescent dye. After heating and cooling, MB probes reform a stem-loop structure which quenches the fluorescent signal from the dye. If a PCR product whose sequence is complementary to the loop sequence is present during the heating/cooling cycle, hybridization of the MB

to one strand of the PCR product will increase the distance between the Dabcyl and the dye, resulting in increased fluorescence.

A schematic of the oligonucleotides used for Digital Amplifications shown in Fig. 1C. Two unmodified oligonucleotides are used as primers for the PCR reaction. Two MB probes, each labeled with a different fluorophore, are used to detect the PCR products. MB-GREEN has a loop region that is complementary to the portion of the WT PCR product that is queried for mutations. Mutations within the corresponding sequence of the PCR product should significantly impede the hybridization of it to the MB probe. MB-RED has a loop region that is complementary to a different portion of the PCR product, one not expected to be mutant. It thus should produce a signal whenever a well contains a PCR product, whether that product is WT or mutant in the region queried by MB-GREEN. Both MB probes are used together to simultaneously detect the presence of a PCR product and its mutational status.

Practical Considerations.

Numerous conditions were optimized to define conditions that could be reproducibly and generally applied. As outlined in Fig. 1A, the first step involves amplification from single template molecules. Most protocols for amplification from small numbers of template molecules use a nesting procedure, wherein a product resulting from one set of primers is used as template in a second reaction employing internal primers. As many applications of digital amplification are expected to require hundreds or thousands of separate amplifications, such nesting would be inconvenient and could lead to contamination problems. Hence, conditions were sought that would achieve robust amplification without nesting. The most important of these conditions involved the use of a polymerase that was activated only after heating and optimized concentrations of dNTP's, primers, buffer components,

and temperature. The conditions specified in Examples 1-3 were defined after individually optimizing each of these components and proved suitable for amplification of several different human genomic DNA sequences. Though the time required for PCR was not particularly long (~2.5 hr), the number of cycles used was high and excessive compared to the number of cycles required to amplify the "average" single template molecule. The large cycle number was necessary because the template in some wells might not begin to be amplified until several PCR cycles had been completed. The large number of cycles ensured that every well (not simply the average well) would generate a substantial and roughly equal amount of PCR product if a template molecule were present within it.

The second step in Fig 1A involves the detection of these PCR products. It was necessary to considerably modify the standard MB probe approach in order for it to function efficiently in Digital Amplification applications. Theoretically, one separate MB probe could be used to detect each specific mutation that might occur within the queried sequence. By inclusion of one MB corresponding to WT sequence and another corresponding to mutant sequence, the nature of the PCR product would be revealed. Though this strategy could obviously be used effectively in some situations, it becomes complex when several different mutations are expected to occur within the same queried sequence. For example, in the *c-Ki-Ras* gene example explored here, twelve different base substitutions resulting in missense mutations could theoretically occur within codons 12 and 13, and at least seven of these are observed in naturally-occurring human cancers. To detect all twelve mutations as well as the WT sequence with individual Molecular Beacons would require 13 different probes. Inclusion of such a large number of MB probes would not only raise the background fluorescence but would be expensive. We therefore attempted to develop a single probe that would react with WT sequences better than any mutant sequence within the

queried sequence. We found that the length of the loop sequence, its melting temperature, and the length and sequence of the stem were each important in determining the efficacy of such probes. Loops ranging from 14 to 26 bases and stems ranging from 4 to 6 bases, as well as numerous sequence variations of both stems and loops, were tested during the optimization procedure. For discrimination between WT and mutant sequences (MB-GREEN probe), we found that a 16 base pair loop, of melting temperature (Tm) 50-51°, and a 4 bp stem, of sequence 5'-CACG-3', were optimal. For MB-RED probes, the same stem, with a 19-20 bp loop of Tm 54-56°, proved optimal. The differences in the loop sizes and melting temperatures between MB-GREEN and MB-RED probes reflected the fact that only the GREEN probe is designed to discriminate between closely related sequences, with a shorter region of homology facilitating such discrimination.

Examples of the ratios obtained in replicate wells containing DNA templates from colorectal tumor cells with mutations of *c-Ki-Ras* are shown in Fig. 2. In this experiment, fifty copies of genomic DNA equivalents were diluted into each well prior to amplification. Each of six tested mutants yielded ratios of RED/GREEN fluorescence that were significantly in excess of the ratio obtained with DNA from normal cells (1.5 to 3.4 in the mutants compared to 1.0 in normal DNA; $p < 0.0001$ in each case, Student's t-Test). The reproducibility of the ratios can be observed in this figure. Direct DNA sequencing of the PCR products used for fluorescence analysis showed that the RED/GREEN ratios were dependent on the relative fraction of mutant genes within the template population (Fig. 2). Thus, the DNA from cells containing one mutant *C-Ki-Ras* allele per every two WT *c-Ki-Ras* allele yielded a RED/GREEN ratio of 1.5 (Gly12Arg mutation) while the cells containing three mutant *c-Ki-Ras* alleles per WT allele exhibited a ratio of 3.4 (Gly12Asp). These data suggested that wells containing only mutant alleles

(no WT) would yield ratios in excess of 3.0, with the exact value dependent on the specific mutation.

Though this mode is the most convenient for many applications, we found it useful to add the MB probes after the PCR-amplification was complete (Fig. 1). This allowed us to use a standard multiwell plate fluorometer to sequentially analyze a large number of multiwell plates containing pre-formed PCR products and bypassed the requirement for multiple real time PCR instruments. Additionally, we found that the fluorescent signals obtained could be considerably enhanced if several cycles of asymmetric, linear amplification were performed in the presence of the MB probes. Asymmetric amplification was achieved by including an excess of a single internal primer (primer INT in Fig. 1C) at the time of addition of the MB probes.

EXAMPLE 5

Analysis of DNA from tumor cells. The principles and practical considerations described above was demonstrated with DNA from two colorectal cancer cell lines, one with a mutation in *c-Ki-Ras* codon 12 and the other in codon 13. Representative examples of the MB-RED fluorescence values obtained are shown in Fig. 3. There was a clear biphasic distribution, with "positive" wells yielding values in excess of 10,000 specific fluorescence units (SFU, as defined in Materials and Methods) and "negative" wells yielding values less than 3500 SFU. Gel electrophoreses of 127 such wells demonstrated that all positive wells, but no negative wells, contained PCR products of the expected size (Fig. 3). The RED/GREEN fluorescence ratios of the positive wells are shown in Fig. 4. Again, a biphasic distribution was observed. In the experiment with the tumor containing a Gly12Asp mutation, 64% of the positive wells exhibited RED/GREEN ratios in excess of 3.0 while the other 36% of the positive wells exhibited ratios ranging from 0.8 to 1.1. In the case of the tumor with the Gly13Asp mutation, 54% of the positive wells exhibited RED/GREEN ratios >3.0 while the other positive wells yielded ratios ranging from 0.9 to 1.1. The PCR products from 16 positive wells were used as sequencing templates (Fig. 4). All the wells yielding a ratio in excess of 3.0 were found to contain mutant c-Ki-Ras fragments of the expected sequence, while WT sequence was found in the other PCR products. The presence of homogeneous WT or mutant sequence confirmed that the amplification products were usually derived from single template molecules. The ratios of WT to mutant PCR products determined from the Digital Amplification assay was also consistent with the fraction of mutant alleles inferred from direct sequence analysis of genomic DNA from the two tumor lines (Fig. 2).

Digital Analysis of DNA from stool. As a more practical example, we analyzed the DNA from stool specimens from colorectal cancer patients. A representative result of such an experiment is illustrated in Fig. 5. From previous analyses of stool specimens from patients whose tumors contained *c-Ki-Ras* gene mutations, we expected that 1% to 10% of the *c-Ki-Ras* genes purified from stool would be mutant. We therefore set up a 384 well Digital Amplification experiment. As positive controls, 48 of the wells contained 25 genome equivalents of DNA (defined in Materials and Methods) from normal cells. Another 48 wells served as negative controls (no DNA template added). The other 288 wells contained an appropriate dilution of stool DNA. MB-RED fluorescence indicated that 102 of these 288 experimental wells contained PCR products (mean +/- s.d. of 47,000 +/- 18,000 SFU) while the other 186 wells did not (2600 +/- 1500 SFU). The RED/GREEN ratios of the 102 positive wells suggested that five contained mutant c-Ki-Ras genes, with ratios ranging from 2.1 to 5.1. The other 97 wells exhibited ratios ranging from 0.7 to 1.2, identical to those observed in the positive control wells. To determine the nature of the mutant *c-Ki-Ras* genes in the five positive wells from stool, the PCR products were directly sequenced. The four wells exhibiting RED/GREEN ratios in excess of 3.0 were completely composed of mutant c-Ki-Ras sequence (Fig. 5B). The sequence of three of these PCR products revealed Gly12Ala mutations (GGT to GCT at codon 12), while the sequence of the fourth indicated a silent C to T transition at the third position of codon 13. This transition presumably resulted from a PCR error during the first productive cycle of amplification from a WT template. The well with a ratio of 2.1 contained a ~1:1 mix of WT and Gly12Ala mutant sequences. Thus 3.9% (4/102) of the *c-Ki-Ras* alleles present in this stool sample contained a Gly12Ala mutation. The mutant alleles in the stool presumably arose from the colorectal cancer of the patient, as direct sequencing of PCR products

generated from DNA of the cancer revealed the identical Gly12Ala mutation (not shown).

CLAIMS

1. A method for detecting a cancer-associated mutant nucleic acid that is present in a patient sample at a low level relative to a corresponding wild-type nucleic acid, the method comprising:

diluting nucleic acids in a biological sample to form a set comprising a plurality of assay samples;

amplifying the nucleic acids in the assay samples to form a population of amplified molecules;

performing an assay on the amplified molecules in each assay sample to determine whether a cancer-associated mutation is present in at least one of the assay samples;

wherein the step of diluting in performed until at least one-fiftieth of the assay samples in the set comprise a number (N) of molecules such that 1/N is larger than a ratio of the mutant nucleic acid to the wild-type nucleic acid required to detect the mutant nucleic acid if it is present in the assay sample.

2. The method of claim 1 wherein the step of diluting is performed until between 0.1 and 0.9 of the assay samples yield an amplification product when subjected to a polymerase chain reaction.
3. The method of claim 1 wherein the step of diluting is performed until all of the assay samples yield an amplification product when subjected to a polymerase chain reaction and each assay sample contains less than 10 nucleic acid template molecules containing a reference genetic sequence.
4. The method of claim 1 wherein the step of diluting is performed until all of the assay samples yield an amplification product when subjected to a polymerase chain reaction and each assay sample contains less than

100 nucleic acid template molecules containing a reference genetic sequence.

5. The method of claim 1 wherein the biological sample is cell-free.
6. The method of claim 1 wherein the number of assay samples within the set is greater than 10.
7. The method of claim 1 wherein the number of assay samples within the set is greater than 50.
8. The method of claim 1 wherein the number of assay samples within the set is greater than 100.
9. The method of claim 1 wherein the number of assay samples within the set is greater than 500.
10. The method of claim 1 wherein the number of assay samples within the set is greater than 1000.
11. The method of claim 1 wherein the step of amplifying and the step of analyzing are performed on assay samples in the same receptacle.
12. The method of claim 1 wherein a molecular beacon probe is used in the step of analyzing, wherein a molecular beacon probe is an oligonucleotide with a stem-loop structure having a photoluminescent dye at one of the 5' or 3' ends and a quenching agent at the opposite 5' or 3' end.
13. The method of claim 1 wherein the step of analyzing employs gel electrophoresis.
14. The method of claim 1 wherein the step of analyzing employs hybridization to at least one nucleic acid probe.
15. The method of claim 1 wherein the step of analyzing employs hybridization to at least two nucleic acid probe.
16. The method of claim 12 wherein two molecular beacon probes are used, each having a different photoluminescent dye.

17. The method of claim 12 wherein the molecular beacon probe detects a wild-type nucleic acid better than a mutant nucleic acid.
18. The method of claim 1 wherein the step of amplifying employs a single pair of primers.
19. The method of claim 1 wherein the step of amplifying employs a polymerase which is activated only after heating.
20. . The method of claim 1 wherein the step of amplifying employs at least 40 cycles of heating and cooling.
21. The method of claim 1 wherein the step of amplifying employs at least 50 cycles of heating and cooling.
22. The method of claim 1 wherein the step of amplifying employs at least 60 cycles of heating and cooling.
23. The method of claim 1 wherein the biological sample is selected from the group consisting of stool, blood, and lymph nodes.
24. The method of claim 1 wherein the biological sample is blood or bone marrow of a leukemia or lymphoma patient who has received anti-cancer therapy.
25. The method of claim 1 wherein the mutant nucleic acid is a translocated allele
26. The method of claim 1 wherein the mutant nucleic acid is within an amplicon which is amplified during neoplastic development.
27. The method of claim 1 wherein the mutant nucleic acid is a rare exon sequence
28. The method of claim 1 wherein the nucleic acids being analyzed comprise cDNA of RNA transcripts.
29. A method for determining the ratio of a selected genetic sequence in a population of genetic sequences from a **blood** sample, comprising the steps of:

diluting nucleic acid template molecules from a **blood** sample to form

a set comprising a plurality of assay samples;

amplifying the template molecules within the assay samples to form a population of amplified molecules in the assay samples of the set;

analyzing the amplified molecules in the assay samples of the set to determine a first number of assay samples which contain the selected genetic sequence and a second number of assay samples which contain a reference genetic sequence;

comparing the first number to the second number to ascertain a ratio which reflects the composition of the **blood** sample.

30. The method of claim 29 wherein the step of amplifying employs **real-time polymerase chain reactions**.

31. The method of claim 30 wherein the real-time polymerase chain reactions comprise a **dual-labeled fluorogenic probe**.

32. The method of claim 29 further comprising the step of :
identifying **an allelic imbalance** based on the ratio ascertained.

33. The method of claim 29 wherein the selected genetic sequences and the reference genetic sequence are **non-polymorphic markers**.

34. The method of claim 29 wherein the selected genetic sequence and the reference genetic sequence are **on distinct chromosomes**.

35. A method for determining the ratio of a selected **non-polymorphic marker** in a population of genetic sequences in a **biological** sample, comprising the steps of:
diluting nucleic acid template molecules in a biological sample to form

a set comprising a plurality of assay samples;

amplifying the template molecules within the assay samples to form a population of amplified molecules in the assay samples of the set;

analyzing the amplified molecules in the assay samples of the set to determine a first number of assay samples which contain the selected **non-polymorphic marker** and a second number of assay samples which contain a reference **non-polymorphic marker, wherein the selected and reference non-polymorphic markers are on distinct chromosomes**;

comparing the first number to the second number to ascertain a ratio which reflects the composition of the **biological** sample; and

identifying **an allelic imbalance** based on the ratio ascertained.

36. The method of claim 35 wherein the biological sample is a blood sample.

37. The method of claim 35 wherein the step of amplifying employs real-time polymerase chain reactions.

38. The method of claim 37 wherein the real-time polymerase chain reactions comprise a dual-labeled fluorogenic probe.

39. A method for determining the ratio of a selected genetic sequence in a population of genetic sequences from a **blood** sample, comprising the steps of:

amplifying template molecules within a set comprising a plurality of assay samples to form a population of amplified molecules in each of the assay samples of the set, wherein the template molecules are obtained from a **blood** sample;

analyzing the amplified molecules in the assay samples of the set to determine a first number of assay samples which contain the selected genetic sequence and a second number of assay samples which contain a reference genetic sequence, wherein at least one-fiftieth of the assay samples in the set comprise a number (N) of molecules such that 1/N is larger than the ratio of selected genetic sequences to total genetic sequences required to determine the presence of the selected genetic sequence;

comparing the first number to the second number to ascertain a ratio which reflects the composition of the **blood** sample.

40. The method of claim 39 wherein the step of amplifying employs **real-time polymerase chain reactions**.

41. The method of claim 40 wherein the real-time polymerase chain reactions comprise a **dual-labeled fluorogenic probe**.

42. The method of claim 39 further comprising the step of :
identifying **an allelic imbalance** based on the ratio ascertained.

43. The method of claim 39 wherein the selected genetic sequences and the reference genetic sequence are **non-polymorphic markers**.

44. The method of claim 39 wherein the selected genetic sequence and the reference genetic sequence are **on distinct chromosomes**.

45. A method for determining the ratio of a selected **non-polymorphic marker** in a population of **non-polymorphic markers** from a **biological** sample, comprising the steps of:
amplifying template molecules within a set comprising a plurality of

assay samples to form a population of amplified molecules in each of the assay samples of the set , wherein the template molecules are obtained from a biological sample;

analyzing the amplified molecules in the assay samples of the set to determine a first number of assay samples which contain the selected **non-polymorphic marker** and a second number of assay samples which contain a reference **non-polymorphic marker**, wherein at least one-fiftieth of the assay samples in the set comprise a number (N) of molecules such that 1/N is larger than the ratio of selected **non-polymorphic marker** to total **non-polymorphic markers** required to determine the presence of the selected **non-polymorphic marker,** wherein the selected genetic sequence and the reference genetic sequence are **on distinct chromosomes**;

comparing the first number to the second number to ascertain a ratio which reflects the composition of the biological sample; and

identifying **an allelic imbalance based on the ratio ascertained.**

46. The method of claim 45 wherein the step of amplifying employs real-time polymerase chain reactions.

47. The method of claim 46 wherein the real-time polymerase chain reactions comprise a dual-labeled fluorogenic probe.

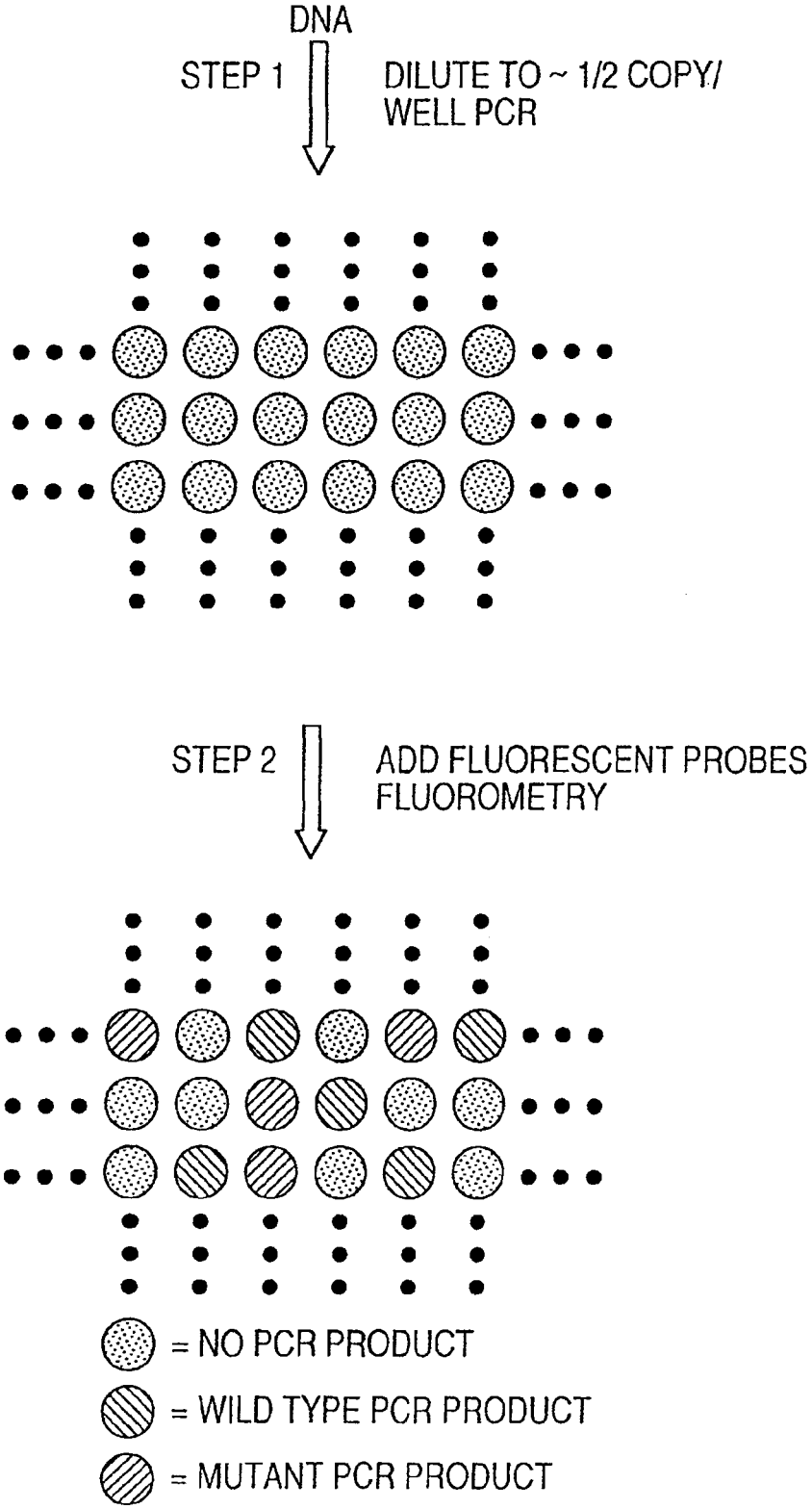
48. The method of claim 45 wherein the biological sample is from blood.

DIGITAL AMPLIFICATION

ABSTRACT

The identification of pre-defined mutations expected to be present in a minor fraction of a cell population is important for a variety of basic research and clinical applications. The exponential, analog nature of the polymerase chain reaction is transformed into a linear, digital signal suitable for this purpose. Single molecules can be isolated by dilution and individually amplified; each product is then separately analyzed for the presence of pre-defined mutations. The process provides a reliable and quantitative measure of the proportion of variant sequences within a DNA sample.

FIG. 1A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STEP 2
ADD FLUORESCENT PROBES FLUOROMETRY
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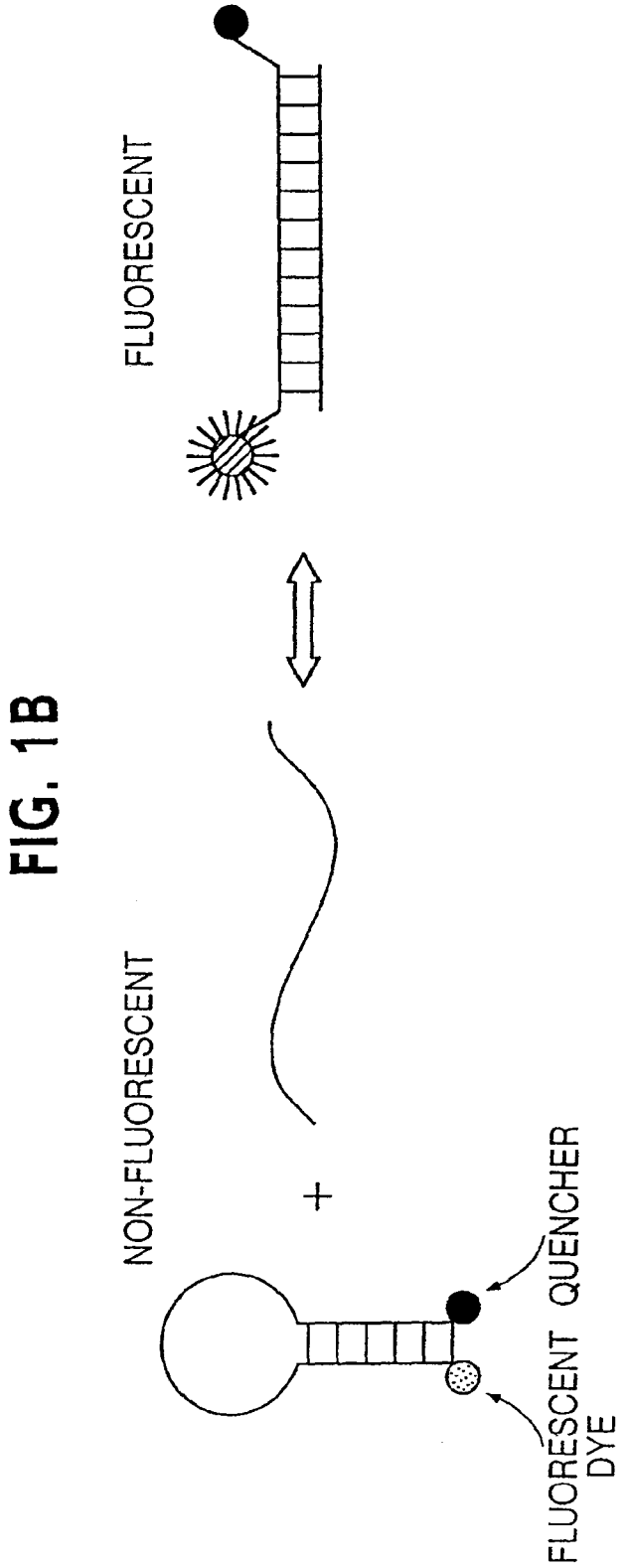
= WILD TYPE PCR PRODUCT
= MUTANT PCR PRODUCT



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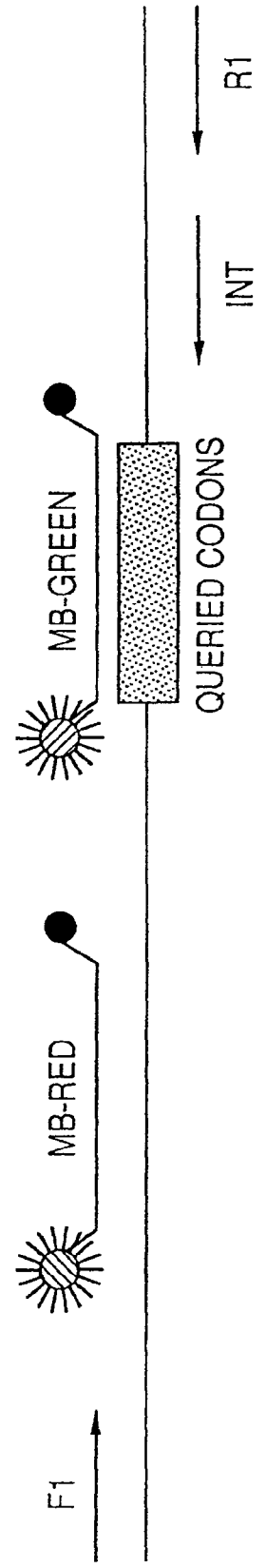
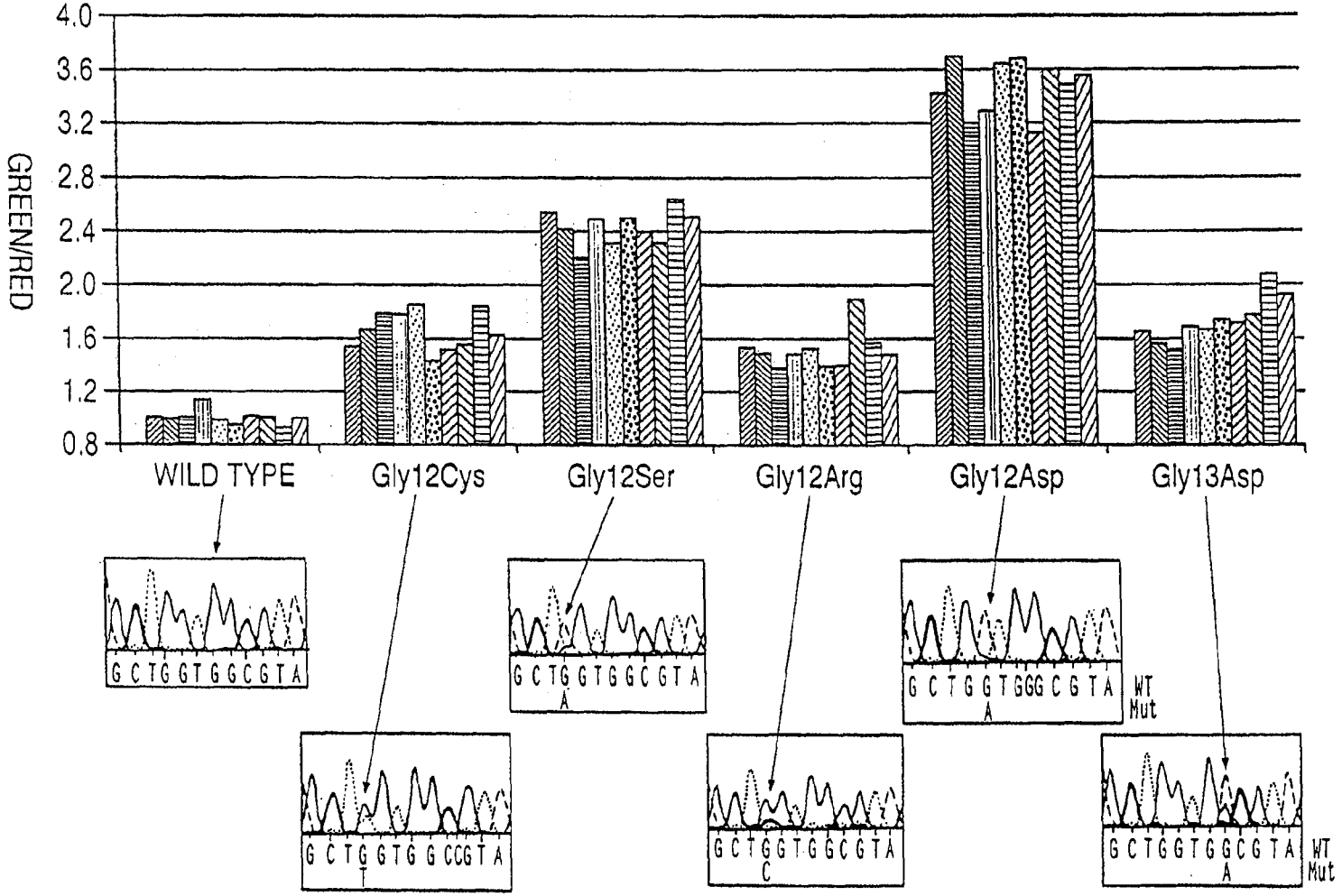


FIG. 1C

FIG. 2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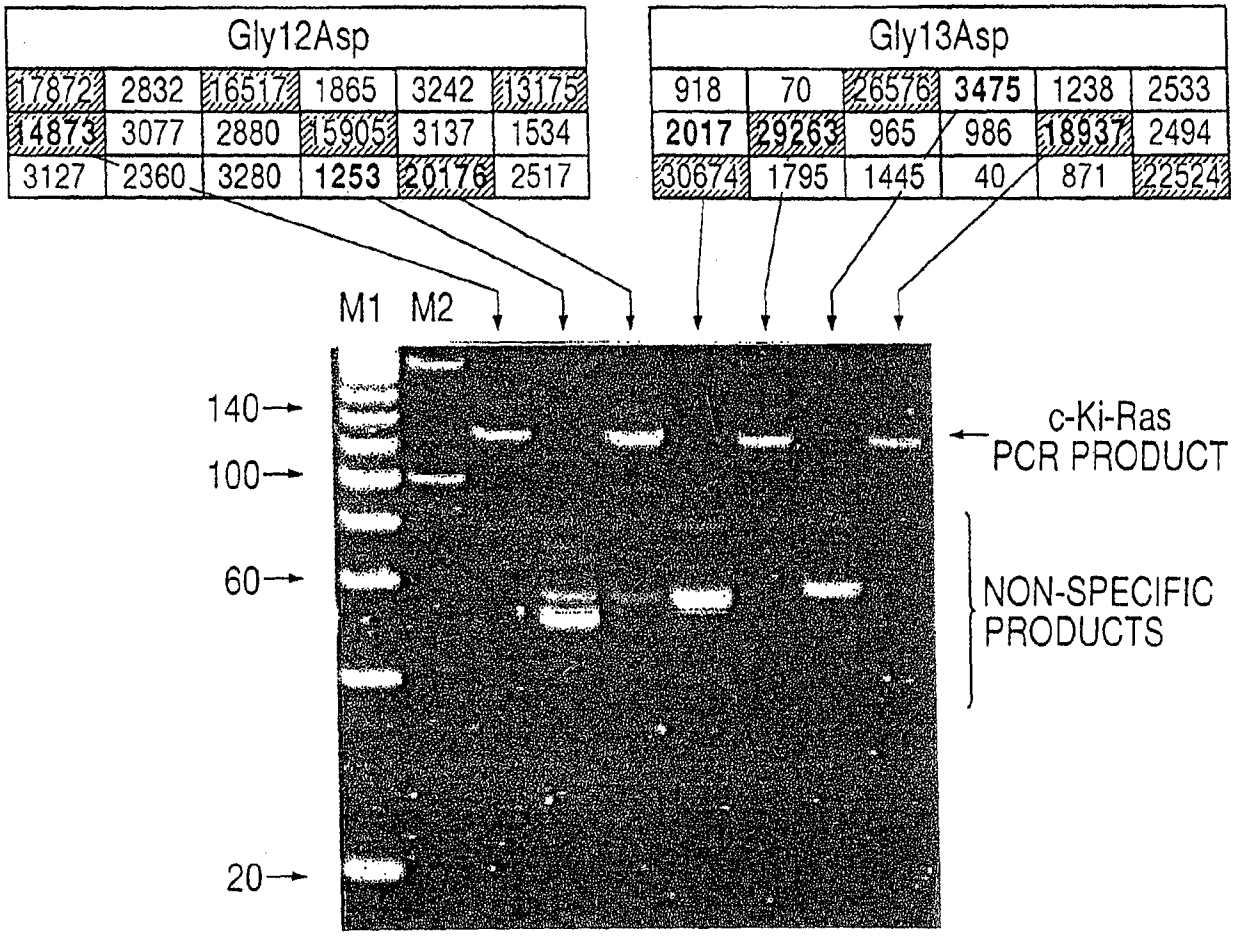
WT
Mut

FIG. 3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PRODUCTS

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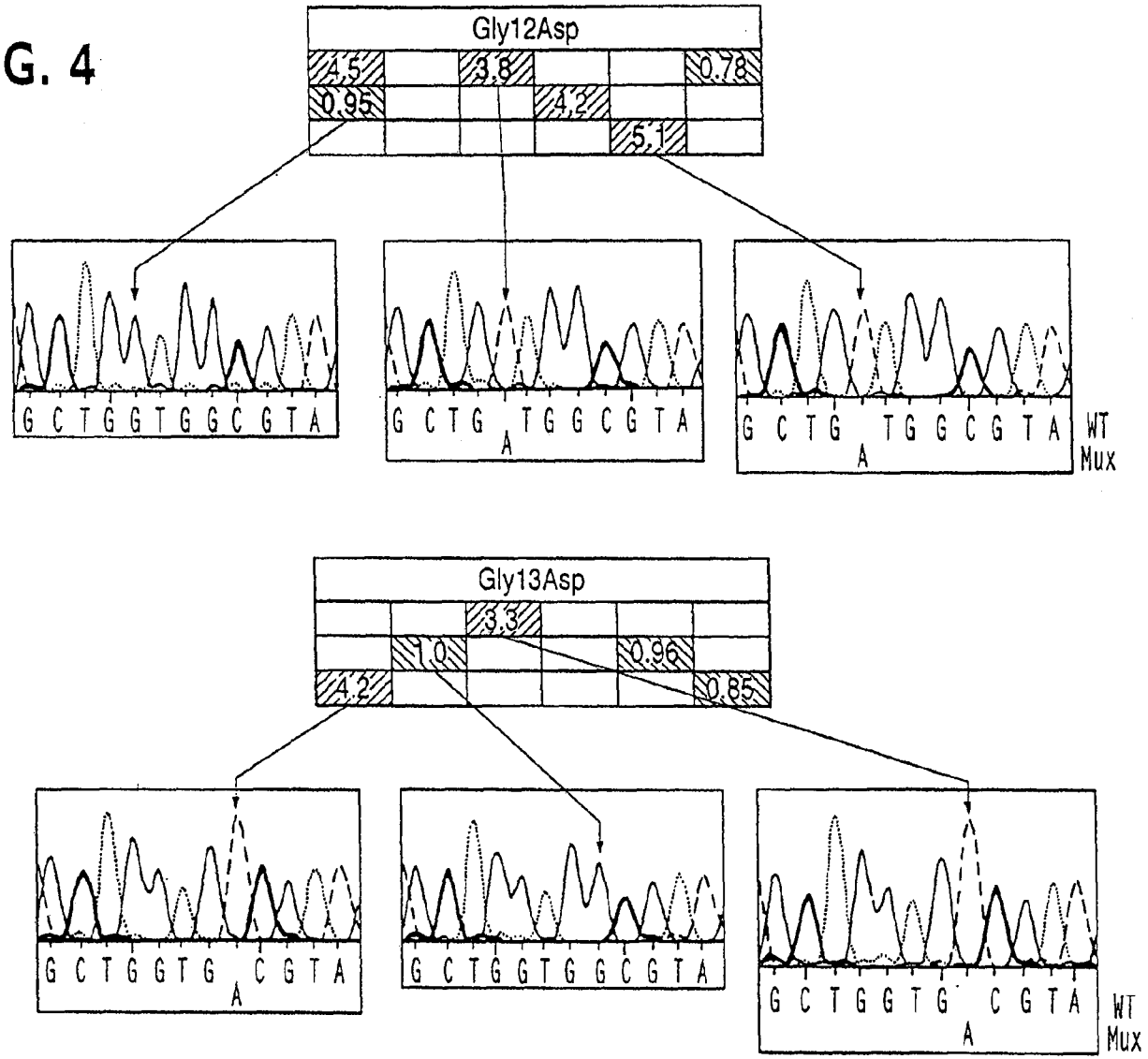
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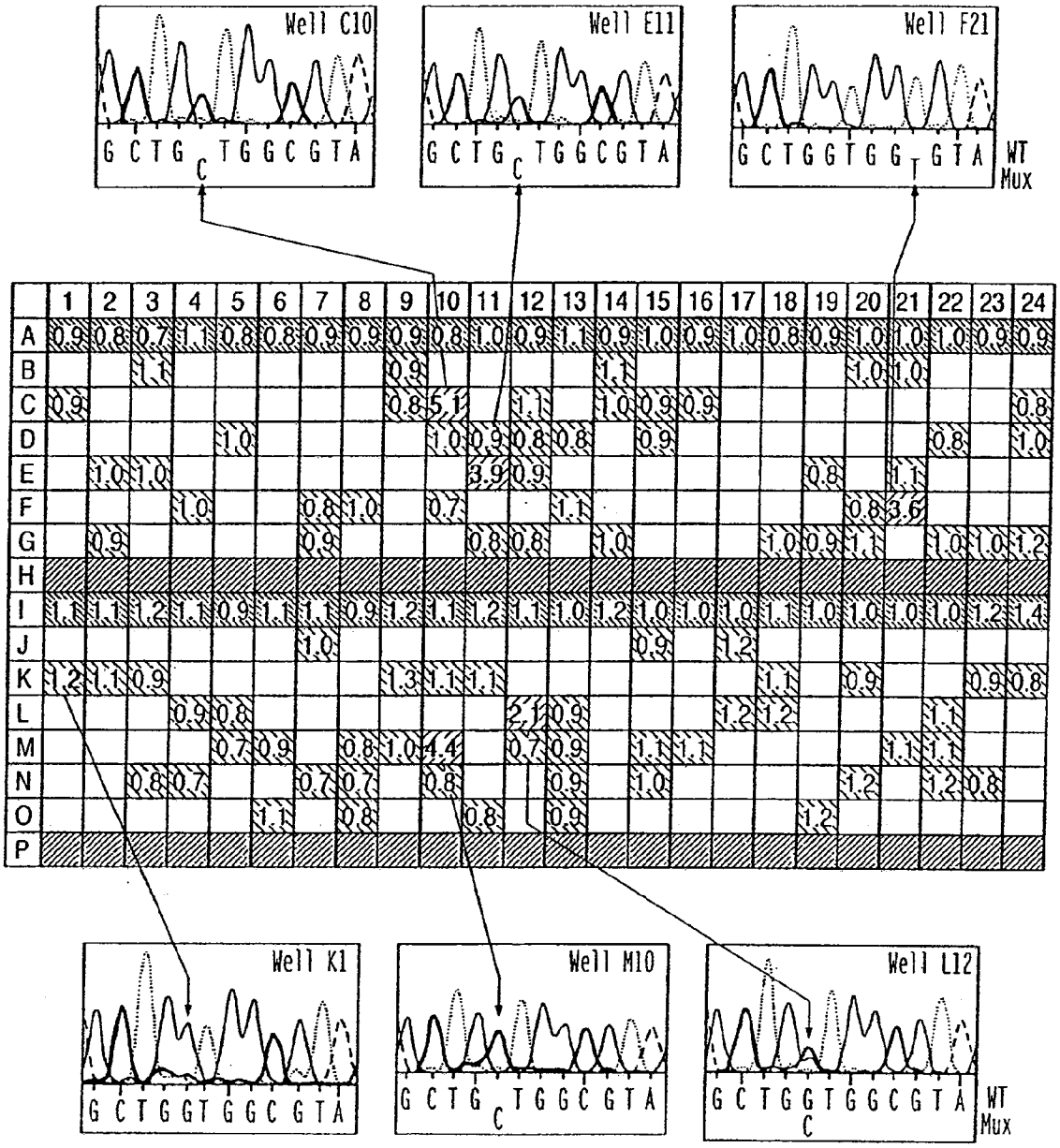
FIG. 5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B 1.1 0.9 1.1 1.0 1.0
C 0.9 0.8 5.1 1.1 1.0 0.9 0.9 0.8
D 1.0 1.0 0.9 0.8 0.8 0.9 0.8 1.0
E 1.0 1.0 3.9 0.9 0.8 1.1
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WT Mux

PATENT

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re Application of	)	Prior Group Art Unit: 1637
	)	
Bert VOGELSTEIN et al	)	Prior Examiner: S. Woolwine
	)	
Serial No. TBD	)	Confirmation No. TBD
	)	
Filed: Herewith	)	Atty. Dkt. No. 001107.00866
	)	
For: DIGITAL AMPLIFICATION	)	

SEQUENCE STATEMENT

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Sir:

Applicants respectfully request that the Patent Office use the computer readable form of the sequence listing submitted on November 14, 2003 in parent Application Serial Number 09/981,356 for examination of the instant application. I believe the contents of the referenced computer readable form and the paper copy of the sequence listing submitted herewith are identical. No new matter is added.

Respectfully submitted,

Date: March 24, 2011

By: /Sarah A. Kagan/
Sarah A. Kagan
Registration No. 32,141

Banner & Witcoff, Ltd.
Customer No. 22907

SEQUENCE LISTING

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Kinzler, Kenneth W.

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PATENT

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re Application of	)	Prior Group Art Unit: 1637
	)	
Bert VOGELSTEIN et al	)	Prior Examiner: Samuel Woolwine
	)	
Continuation Application of	)	Confirmation No. TBD
Serial No. 12/617,368	)	
	)	Atty. Dkt. No. 001107.00866
Filed: Herewith	)	
	)	
For: DIGITAL AMPLIFICATION	)	

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In accordance with 37 C.F.R. § 1.97, enclosed is a PTO Form 1449 listing documents for consideration by the Examiner in the subject application. Copies of the cited references were submitted in parent Application No. 12/617,368 or were provided by the Examiner attached to an office action. No fee is believed to be due to ensure consideration and entry of the cited documents by the Examiner. However, if a fee is deemed necessary, the Commissioner is authorized to charge our Deposit Account No. 19-0733.

Respectfully submitted,

By: /Sarah A. Kagan/
Sarah A. Kagan
Registration No. 32,141

Date: March 24, 2011

Banner & Witcoff, Ltd.
Customer No. 22907

PTO/SB/08a (01-10)
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INFORMATION DISCLOSURE STATEMENT BY APPLICANT

(Not for submission under 37 CFR 1.99)

Application Number	
Filing Date	2011-03-16
First Named Inventor	Bert Vogelstein et al.
Art Unit	
Examiner Name	
Attorney Docket Number	001107.00866

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Examiner Initial*	Cite No	Patent Number	Kind Code[1]	Issue Date	Name of Patentee or Applicant of cited Document	Pages,Columns,Lines where Relevant Passages or Relevant Figures Appear
	1	5213961	A	1993-05-15	Bunn et al.	
	2	5736333	A	1998-04-07	Livak et al.	
	3	5518901	A	1996-05-21	Murtagh	
	4	5804383	A	1998-09-08	Gruenert et al.	
	5	5858663	A	1999-01-12	Nisson et al.	
	6	5670325	A	1997-09-23	Lapidus et al.	
	7	6037130	A	2000-03-14	Tyagi et al.	
	8	5925517	A	1999-07-20	Tyagi et al.	

INFORMATION DISCLOSURE STATEMENT BY APPLICANT (Not for submission under 37 CFR 1.99)		
	Application Number	
	Filing Date	2011-03-16
	First Named Inventor	Bert Vogelstein et al.
	Art Unit	
	Examiner Name	
	Attorney Docket Number	001107.00866

	9	5928870	A	1999-07-27	Lapidus et al.	
	10	6020137	A	2000-02-01	Lapidus et al.	
	11	6143496	A	2000-11-07	Brown et al.	
	12	6291163	B1	2001-09-18	Sidransky	

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	1	95/13399	WO	A1	1995-05-18			☐
	2	99/13113	WO	A1	1999-03-18			☐

INFORMATION DISCLOSURE STATEMENT BY APPLICANT (Not for submission under 37 CFR 1.99)		
	Application Number	
	Filing Date	2011-03-16
	First Named Inventor	Bert Vogelstein et al.
	Art Unit	
	Examiner Name	
	Attorney Docket Number	001107.00866

	3	0643140	EP	A1	1995-03-15	Canon Kabushiki Kaisha		☐

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	1	LOUGHLIN ET AL., "Association of the Interleukin-1 Gene Cluster on Chromosome 2q13 With Knee Osteoarthritis," Arthritis & Rheumatism, June 2002, 46(6):1519-1527	☐
	2	P. J. SYKES, "Quantitation of Targets for PCR by Use of Limiting Dilution," BioTechniques, 1992, Vol. 13, No. 3, pp. 444-449	☐
	3	A. PIATEK ET AL., "Molecular Beacon Sequence Analysis for Detecting Drug Resistance in Mycobacterium Tuberculosis," Nature Biotechnology, April 1998, Vol. 16, No. 4. pp. 359-363	☐
	4	S. TYAGI ET AL., "Multicolor Molecular Beacons for allele discrimination," Nature Biotechnology, January 1998, Vol. 16, No. 1, pp. 303-308	☐
	5	J. A.M. VET ET AL., "Multilex Detection of Four Pathogenic Retroviruses Using Molecular Beacons," Proceedings of the National Academy of Sciences of the United States", May 25, 1999, Vol. 96, No. 11, pp. 6394-6399	☐
	6	S. TYAGI ET AL., "Molecular Beacons: probes that Fluoresce Upon Hybridization," Nature Biotechnology, 1996, Vol. 14, No. 3, pp. 303-308	☐
	7	W. P. HALFORD ET AL., "The Inherent Quantitative Capacity of the Reverse Transcription-Polymerase Chain Reaction," Analytical Biochemistry, January 15, 1999, Vol. 266, No. 2, pp. 181-191	☐
	8	B. VOGELSTEIN ET AL., "Digital PCR," Proceedings of the National Academy of Sciences of the United States, August 3, 1999, Vol. 96, No. 16, pp. 9236-9241	☐

INFORMATION DISCLOSURE STATEMENT BY APPLICANT (Not for submission under 37 CFR 1.99)		
	Application Number	
	Filing Date	2011-03-16
	First Named Inventor	Bert Vogelstein et al.
	Art Unit	
	Examiner Name	
	Attorney Docket Number	001107.00866

	9	K. D.E. EVERETT ET AL., "Identification of Nine Species of the Chlamydiaceae Using PCR-RFLP," Int. J. Syst. Bacteriol., April 1999, Vol. 49, No. 2, pp. 803-813	☐
	10	D. G. MONCKTON ET AL., "Minisatellite "Isoallele" Discrimination in Pseudohomozygotes by Single Molecule PCR and Variant Repeat Mapping," Genomics, 1991, Vol. 11, pp. 465-467	☐
	11	G. RUANO ET AL., "Haplotype of Multiple Polymorphisms Resolved by Enzymatic Amplification of Single DNA Molecules," Proc. National Science USA, 1990, pp. 6296-6300	☐
	12	W. NAVIDI ET AL., "Using PCR in Preimplantation Genetic Disease Diagnosis," Human Reproduction, 1991, Vol. 6, No. 6, pp. 836-849	☐
	13	H. LI ET AL., "Amplification and Analysis of DNA Sequences in Single Human Sperm and Diploid Cells," Nature, September 29, 1988, Vol. 335, pp. 414-417	☐
	14	L. ZHANG ET AL., "Whole Genome Amplification from a Single Cell: Implications for Genetic Analysism," Proc. National Science USA, July 1992, Vol. 89, pp. 5847-5851	☐
	15	D. SIDRANSKY ET AL., "Clonal Expansion of p53 Mutant Cells is Associated with Brain Tumour Progression," Nature, February 27, 1992, pp. 846-847	☐
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	18	P. M. LIZARDI ET AL., "Mutation Detection and Single-Molecule Counting Using Isothermal Rolling-Circle Amplification," Nature Genetics, July 1998, Vol. 19, pp. 225-232	☐
	19	R. PARSONS ET AL., "Mismatch Repair Deficiency in Phenotypically Normal Human Cells," Science, May 5, 1995, Vol. 268, pp. 738-740	☐

INFORMATION DISCLOSURE STATEMENT BY APPLICANT (Not for submission under 37 CFR 1.99)		
	Application Number	
	Filing Date	2011-03-16
	First Named Inventor	Bert Vogelstein et al.
	Art Unit	
	Examiner Name	
	Attorney Docket Number	001107.00866

	20	MARRAS ET AL., "Multiplex Detection of Single-Nucleotide Variations Using Molecular Beacons," Genetic Analysis: Biomolecular Engineering, Feb. 1999, Vo. 14, pp. 151-156	☐
	21	WHITCOMB ET AL., "Detection of PCR Products Using Self-Probing Amplicons and Fluorescence," Nature Biotechnology, August 1999, Vol. 17, pp. 804-807	☐
	22	M.J. BRISCO ET AL., "Detection and Quantitation of Neoplastic Cells in Acute Lymphoblastic Leukemia, by Use of the Polymerase Chain Reaction," British Journal of Haematology, 1991, Vol. 79, pp. 211-217	☐
	23	M. J. BRISCO ET AL., "Outcome Prediction in Childhood Acute Lymphoblastic Leukemia by Molecular Quantification of Residual Disease at the End of Induction," The Lancet, January 22, 1994, Vol. 343, pp. 196-200	☐
	24	Notice of Reasons for Rejection dispatched April 28, 2010 in Japanese Application No. 2001-513641 and English translation thereof.	☐
	25	Stephens, J. Clairborne, et al. "Theoretical underpinning of the Single-Molecular-Dilution (SMD) Method of Direct Haplotype Resolution," Am. J. Hum. Gen., Vol. 46, pp. 1149-1155 (1990).	☐
	26	NEWTON, PCR Essential Data, pages 51-52, 1995	☐
	27	Office Action dated June 11, 2010, in co-pending application 11/709,742	☐
	28	Office Action dated December 29, 2009 in co-pending application 11/709,742	☐
	29	Office Action dated September 18, 2009 in co-pending application 11/709,742	☐
	30	Office Action dated June 5, 2009 in co-pending application 11/709,742	☐

INFORMATION DISCLOSURE STATEMENT BY APPLICANT (Not for submission under 37 CFR 1.99)		
	Application Number	
	Filing Date	2011-03-16
	First Named Inventor	Bert Vogelstein et al.
	Art Unit	
	Examiner Name	
	Attorney Docket Number	001107.00866

If you wish to add additional non-patent literature document citation information please click the Add button Add			
EXAMINER SIGNATURE			
Examiner Signature		Date Considered	

*EXAMINER: Initial if reference considered, whether or not citation is in conformance with MPEP 609. Draw line through a citation if not in conformance and not considered. Include copy of this form with next communication to applicant.

[1] See Kind Codes of USPTO Patent Documents at www.USPTO.GOV or MPEP 901.04. [2] Enter office that issued the document, by the two-letter code (WIPO Standard ST.3). [3] For Japanese patent documents, the indication of the year of the reign of the Emperor must precede the serial number of the patent document. [4] Kind of document by the appropriate symbols as indicated on the document under WIPO Standard ST.16 if possible. [5] Applicant is to place a check mark here if English language translation is attached.

INFORMATION DISCLOSURE STATEMENT BY APPLICANT (Not for submission under 37 CFR 1.99)		
	Application Number	
	Filing Date	2011-03-16
	First Named Inventor	Bert Vogelstein et al.
	Art Unit	
	Examiner Name	
	Attorney Docket Number	001107.00866

CERTIFICATION STATEMENT

Please see 37 CFR 1.97 and 1.98 to make the appropriate selection(s):

☐ That each item of information contained in the 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 the information disclosure statement. See 37 CFR 1.97(e)(1).

OR

☐ That no item of information contained in the information disclosure statement was cited in a communication from a foreign patent office in a counterpart foreign application, and, to the knowledge of the person signing the certification after making reasonable inquiry, no item of information contained in the information disclosure statement was known to any individual designated in 37 CFR 1.56(c) more than three months prior to the filing of the information disclosure statement. See 37 CFR 1.97(e)(2).

☐ See attached certification statement.

☐ Fee set forth in 37 CFR 1.17 (p) has been submitted herewith.

☒ None

SIGNATURE

A signature of the applicant or representative is required in accordance with CFR 1.33, 10.18. Please see CFR 1.4(d) for the form of the signature.

Signature	/Sarah A. Kagan/	Date (YYYY-MM-DD)	2011-03-24
Name/Print	Sarah A. Kagan	Registration Number	32141

This collection of information is required by 37 CFR 1.97 and 1.98. 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 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: Commissioner for Patents, P.O. Box 1450, Alexandria, VA 22313-1450.**

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

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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.

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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.

Electronic Patent Application Fee Transmittal

Application Number:	
Filing Date:	
Title of Invention:	DIGITAL AMPLIFICATION
First Named Inventor/Applicant Name:	Bert VOGELSTEIN
Filer:	Sarah Anne Kagan./Daphne Cashion
Attorney Docket Number:	001107.00866

Filed as Large Entity

Utility under 35 USC 111(a) Filing Fees

Description	Fee Code	Quantity	Amount	Sub-Total in USD($)
Basic Filing:				
Utility application filing	1011	1	330	330
Utility Search Fee	1111	1	540	540
Utility Examination Fee	1311	1	220	220
Pages:				
Claims:				
Miscellaneous-Filing:				
Petition:				
Patent-Appeals-and-Interference:				

Description	Fee Code	Quantity	Amount	Sub-Total in USD($)
Post-Allowance-and-Post-Issuance:				
Extension-of-Time:				
Miscellaneous:				
			Total in USD ($)	**1090**

Electronic Acknowledgement Receipt

EFS ID:	9733948
Application Number:	13071105
International Application Number:	
Confirmation Number:	3361
Title of Invention:	DIGITAL AMPLIFICATION
First Named Inventor/Applicant Name:	Bert VOGELSTEIN
Customer Number:	22907
Filer:	Sarah Anne Kagan./Daphne Cashion
Filer Authorized By:	Sarah Anne Kagan.
Attorney Docket Number:	001107.00866
Receipt Date:	24-MAR-2011
Filing Date:	
Time Stamp:	16:36:41
Application Type:	Utility under 35 USC 111(a)

Payment information:

Submitted with Payment	yes
Payment Type	Deposit Account
Payment was successfully received in RAM	$1090
RAM confirmation Number	3277
Deposit Account	190733
Authorized User	

File Listing:

Document Number	Document Description	File Name	File Size(Bytes)/ Message Digest	Multi Part /.zip	Pages (if appl.)

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Information:					
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Warnings:					
Information:					
3	Oath or Declaration filed	001107_00866_Declaration_03_24_2011.PDF	115301 09f9f20aaf9b4a8fa261f79acb28f4a42db123ad	no	2
Warnings:					
Information:					
4	Power of Attorney	001107_00866_Recognition_of_Practitioners_03_24_2011.pdf	77950 04bd3bcf00450857c98b6a79ce7e449392cdc660	no	1
Warnings:					
Information:					
5	Specification	001107_00866_Specification_03_24_2011.pdf	177734 80af79bca00110ec5014249b1f32083e1328124b	no	30
Warnings:					
Information:					
6	Drawings-only black and white line drawings	001107_00866_Drawings_03_24_2011.pdf	354379 b43fcd23a661df79f0e971b83863b27361bd1dd9	no	7
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9	Transmittal Letter	001107_00866_Information_Disclosure_Statement_Transmittal_03_24_2011.pdf	59761 bddaca05e124831ec0d3fc9044cfe59c9ab4214e	no	1
Warnings:					
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10	Information Disclosure Statement (IDS) Filed (SB/08)	001107_00866_Information_Disclosure_Statement_03_24_2011.PDF	617659 86d90511251062e6235e599c7682426e19cb9552	no	8
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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.

RAW SEQUENCE LISTING

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Kinzler, Kenneth W.

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United States Patent and Trademark Office
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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
13/071,105	03/24/2011		1090	001107.00866	48	5

CONFIRMATION NO. 3361

22907
BANNER & WITCOFF, LTD.
1100 13th STREET, N.W.
SUITE 1200
WASHINGTON, DC 20005-4051

FILING RECEIPT

OC000000047240067

Date Mailed: 05/09/2011

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**

Applicant(s)

Bert VOGELSTEIN, Baltimore, MD;
Kenneth W. KINZLER, Baltimore, MD;

Assignment For Published Patent Application

THE JOHNS HOPKINS UNIVERSITY, Baltimore, MD

Power of Attorney:

Dale Hoscheit--19090
Joseph Skerpon--29864
William Fisher--32133
Sarah Kagan--32141
Lisa Hemmendinger--42653

Domestic Priority data as claimed by applicant

This application is a CON of 12/617,368 11/12/2009 PAT 7,915,015
which is a CON of 11/709,742 02/23/2007 PAT 7,824,889
which is a CON of 10/828,295 04/21/2004 ABN
which is a DIV of 09/981,356 10/12/2001 PAT 6,753,147
which is a CON of 09/613,826 07/11/2000 PAT 6,440,706
which claims benefit of 60/146,792 08/02/1999

Foreign Applications (You may be eligible to benefit from the **Patent Prosecution Highway** program at the USPTO. Please see http://www.uspto.gov for more information.)

If Required, Foreign Filing License Granted: 04/19/2011

The country code and number of your priority application, to be used for filing abroad under the Paris Convention, is **US 13/071,105**

Projected Publication Date: 08/18/2011

Non-Publication Request: No

Early Publication Request: No
Title

Digital Amplification

Preliminary Class

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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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Title 35, United States Code, Section 184

Title 37, Code of Federal Regulations, 5.11 & 5.15

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NOT GRANTED

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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
13/071,105	03/24/2011	Bert VOGELSTEIN	001107.00866

CONFIRMATION NO. 3361

22907
BANNER & WITCOFF, LTD.
1100 13th STREET, N.W.
SUITE 1200
WASHINGTON, DC 20005-4051

FORMALITIES LETTER

OC000000047240068

Date Mailed: 05/09/2011

NOTICE TO FILE MISSING PARTS OF NONPROVISIONAL APPLICATION

FILED UNDER 37 CFR 1.53(b)

Filing Date Granted

Items Required To Avoid Abandonment:

An application number and filing date have been accorded to this application. The item(s) indicated below, however, are missing.

Applicant is given **TWO MONTHS** from the date of this Notice within which to file all required items below to avoid abandonment. Extensions of time may be obtained by filing a petition accompanied by the extension fee under the provisions of 37 CFR 1.136(a).

- Additional claim fees of $**1896** as a non-small entity, including any required multiple dependent claim fee, are required. Applicant must submit the additional claim fees or cancel the additional claims for which fees are due.

SUMMARY OF FEES DUE:

Total fee(s) required within **TWO MONTHS** from the date of this Notice is $**1896** for a non-small entity
- Total additional claim fee(s) for this application is $**1896**
  - $**440** for **2** independent claims over 3.
  - $**1456** for **28** total claims over 20.

Replies should be mailed to:

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Commissioner for Patents
P.O. Box 1450
Alexandria VA 22313-1450

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/mhteklu/

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: 13/071,105

APPLICATION AS FILED - PART I

FOR	(Column 1) NUMBER FILED	(Column 2) NUMBER EXTRA	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	330
SEARCH FEE (37 CFR 1.16(k), (i), or (m))	N/A	N/A	N/A			N/A	540
EXAMINATION FEE (37 CFR 1.16(o), (p), or (q))	N/A	N/A	N/A			N/A	220
TOTAL CLAIMS (37 CFR 1.16(i))	48 minus 20 =	* 28			OR	x 52 =	1456
INDEPENDENT CLAIMS (37 CFR 1.16(h))	5 minus 3 =	* 2				x 220 =	440
APPLICATION SIZE FEE (37 CFR 1.16(s))	If the specification and drawings exceed 100 sheets of paper, the application size fee due is $270 ($135 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
* If the difference in column 1 is less than zero, enter "0" in column 2.			TOTAL			TOTAL	2986

APPLICATION AS AMENDED - PART II

		(Column 1) CLAIMS REMAINING AFTER AMENDMENT		(Column 2) HIGHEST NUMBER PREVIOUSLY PAID FOR	(Column 3) PRESENT EXTRA	SMALL ENTITY RATE($)	SMALL ENTITY ADDITIONAL FEE($)	OR	OTHER THAN SMALL ENTITY RATE($)	OTHER THAN SMALL ENTITY ADDITIONAL FEE($)
AMENDMENT A	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	

		(Column 1) CLAIMS REMAINING AFTER AMENDMENT		(Column 2) HIGHEST NUMBER PREVIOUSLY PAID FOR	(Column 3) PRESENT EXTRA	SMALL ENTITY RATE($)	SMALL ENTITY ADDITIONAL FEE($)	OR	OTHER THAN SMALL ENTITY RATE($)	OTHER THAN SMALL ENTITY ADDITIONAL FEE($)
AMENDMENT B	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.

UTILITY PATENT

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re Application of:	)	Group Art Unit: TBA
	)	
Bert VOGELSTEIN et al.	)	Docket No. 001107.00866
	)	
Serial No. 13/071,105	)	Confirmation No: 3361
	)	
Filed: March 24, 2011	)	Examiner: TBA

For: DIGITAL AMPLIFICATION

RESPONSE TO NOTICE TO FILE MISSING PARTS OF NONPROVISIONAL APPLICATION

U.S. Patent and Trademark Office
Customer Service Window
Randolph Building, Mail Stop: Missing Parts
401 Dulany Street
Alexandria, VA 22314

Dear Sir:

In response to the Notice to File Missing Parts of Non-provisional Application under 37 C.F.R. §1.53(b), dated May 9, 2011, applicant submits the fees due. The fees are calculated as follows:

2 Independent claims over 3	$440.00
28 total claims over 20	$1456.00
Total Fees	**$1896.00**

We believe that all Patent and Trademark Office requirements have now been fully met and it we respectfully request that the above-identified patent application be forwarded for examination.

Please charge the filing of this paper and any additional fee, which may be associated to our Deposit Account No. 19-0733.

Respectfully submitted,

By: /Sarah A. Kagan/
Sarah A. Kagan
Registration. No. 32,141

Banner & Witcoff, Ltd.
1100 13th Street, N.W., Suite 1200
Washington, D.C. 20005-4051
(202) 824-3000

Dated: July 8, 2011

Electronic Patent Application Fee Transmittal

Application Number:	13071105
Filing Date:	24-Mar-2011
Title of Invention:	Digital Amplification
First Named Inventor/Applicant Name:	Bert VOGELSTEIN
Filer:	Sarah Anne Kagan./Daphne Cashion
Attorney Docket Number:	001107.00866

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:				
Claims in excess of 20	1202	28	52	1456
Independent claims in excess of 3	1201	2	220	440
Miscellaneous-Filing:				
Petition:				
Patent-Appeals-and-Interference:				
Post-Allowance-and-Post-Issuance:				

Description	Fee Code	Quantity	Amount	Sub-Total in USD($)
Extension-of-Time:				
Miscellaneous:				
			Total in USD ($)	**1896**

Electronic Acknowledgement Receipt

EFS ID:	10480321
Application Number:	13071105
International Application Number:	
Confirmation Number:	3361
Title of Invention:	Digital Amplification
First Named Inventor/Applicant Name:	Bert VOGELSTEIN
Customer Number:	22907
Filer:	Sarah Anne Kagan./Daphne Cashion
Filer Authorized By:	Sarah Anne Kagan.
Attorney Docket Number:	001107.00866
Receipt Date:	08-JUL-2011
Filing Date:	24-MAR-2011
Time Stamp:	15:24:33
Application Type:	Utility under 35 USC 111(a)

Payment information:

Submitted with Payment	yes
Payment Type	Deposit Account
Payment was successfully received in RAM	$1896
RAM confirmation Number	11335
Deposit Account	190733
Authorized User	

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

Charge any Additional Fees required under 37 C.F.R. Section 1.21 (Miscellaneous fees and charges)

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	001107_00866_Response_to_Notice_to_File_Missing_Parts_07_08_2011.pdf	69225 66f3abdce50127e06f04ee09626a2f03c50303b1	no	2
Warnings:					
Information:					
2	Fee Worksheet (SB06)	fee-info.pdf	31711 187f04b1ad78c83f4eb09261dd87d763eee412d8	no	2
Warnings:					
Information:					
		Total Files Size (in bytes):	100936		

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.

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Alexandria, Virginia 22313-1450
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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
13/071,105	03/24/2011	1634	2986	001107.00866	48	5

CONFIRMATION NO. 3361

UPDATED FILING RECEIPT

22907
BANNER & WITCOFF, LTD.
1100 13th STREET, N.W.
SUITE 1200
WASHINGTON, DC 20005-4051

OC000000048749107

Date Mailed: 07/15/2011

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**

Applicant(s)

Bert VOGELSTEIN, Baltimore, MD;
Kenneth W. KINZLER, Baltimore, MD;

Assignment For Published Patent Application

THE JOHNS HOPKINS UNIVERSITY, Baltimore, MD

Power of Attorney:

Dale Hoscheit--19090
Joseph Skerpon--29864
William Fisher--32133
Sarah Kagan--32141
Lisa Hemmendinger--42653

Domestic Priority data as claimed by applicant

This application is a CON of 12/617,368 11/12/2009 PAT 7,915,015
which is a CON of 11/709,742 02/23/2007 PAT 7,824,889
which is a CON of 10/828,295 04/21/2004 ABN
which is a DIV of 09/981,356 10/12/2001 PAT 6,753,147
which is a CON of 09/613,826 07/11/2000 PAT 6,440,706
which claims benefit of 60/146,792 08/02/1999

Foreign Applications (You may be eligible to benefit from the **Patent Prosecution Highway** program at the USPTO. Please see http://www.uspto.gov for more information.)

If Required, Foreign Filing License Granted: 04/19/2011

The country code and number of your priority application, to be used for filing abroad under the Paris Convention, is **US 13/071,105**

Projected Publication Date: 08/18/2011

Non-Publication Request: No

Early Publication Request: No

Title

Digital Amplification

Preliminary Class

435

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 (1-866-999-4158).

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: 13/071,105

APPLICATION AS FILED - PART I

FOR	(Column 1) NUMBER FILED	(Column 2) NUMBER EXTRA	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	330
SEARCH FEE (37 CFR 1.16(k), (i), or (m))	N/A	N/A	N/A			N/A	540
EXAMINATION FEE (37 CFR 1.16(o), (p), or (q))	N/A	N/A	N/A			N/A	220
TOTAL CLAIMS (37 CFR 1.16(i))	48 minus 20 =	* 28			OR	x 52 =	1456
INDEPENDENT CLAIMS (37 CFR 1.16(h))	5 minus 3 =	* 2				x 220 =	440
APPLICATION SIZE FEE (37 CFR 1.16(s))	If the specification and drawings exceed 100 sheets of paper, the application size fee due is $270 ($135 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
* If the difference in column 1 is less than zero, enter "0" in column 2.			TOTAL			TOTAL	2986

APPLICATION AS AMENDED - PART II

		(Column 1) CLAIMS REMAINING AFTER AMENDMENT		(Column 2) HIGHEST NUMBER PREVIOUSLY PAID FOR	(Column 3) PRESENT EXTRA	SMALL ENTITY RATE($)	SMALL ENTITY ADDITIONAL FEE($)	OR	OTHER THAN SMALL ENTITY RATE($)	OTHER THAN SMALL ENTITY ADDITIONAL FEE($)
AMENDMENT A	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	

		(Column 1) CLAIMS REMAINING AFTER AMENDMENT		(Column 2) HIGHEST NUMBER PREVIOUSLY PAID FOR	(Column 3) PRESENT EXTRA	RATE($)	ADDITIONAL FEE($)	OR	RATE($)	ADDITIONAL FEE($)
AMENDMENT B	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".

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APPLICATION NUMBER	FILING OR 371(C) DATE	FIRST NAMED APPLICANT	ATTY. DOCKET NO./TITLE
13/071,105	03/24/2011	Bert VOGELSTEIN	001107.00866

CONFIRMATION NO. 3361

22907
BANNER & WITCOFF, LTD.
1100 13th STREET, N.W.
SUITE 1200
WASHINGTON, DC 20005-4051

PUBLICATION NOTICE

OC000000049394257

Title: Digital Amplification

Publication No.US-2011-0201004-A1
Publication Date:08/18/2011

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

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

APPLICATION NUMBER	PATENT NUMBER	GROUP ART UNIT	FILE WRAPPER LOCATION
13/071,105		1637	

OC000000050585019

Correspondence Address/Fee Address Change

The following fields have been set to Customer Number 11332 on 10/24/2011

- **Correspondence Address**

The address of record for Customer Number 11332 is:

11332
Banner & Witcoff, Ltd.
Attorneys for client 001107
1100 13th Street N.W.
Suite 1200
Washington, DC 20005-4051

PART 1 - ATTORNEY/APPLICANT COPY
page 1 of 1

UTILITY PATENT

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re Application of:	)	Group Art Unit: 1637
	)	
Bert VOGELSTEIN et al.	)	Docket No. 001107.00866
	)	
Serial No. 13/071,105	)	Confirmation No: 3361
	)	
Filed: March 24, 2011	)	Examiner: Woolwine, Samuel C.

For: DIGITAL AMPLIFICATION

PRELIMINARY AMENDMENT

U.S. Patent and Trademark Office
Customer Service Window
Randolph Building, Mail Stop: Missing Parts
401 Dulany Street
Alexandria, VA 22314

Dear Sir:

Please enter the following amendment to the application before examination commences. Should any additional fees be required to enter this amendment, please charge our deposit account no. 19-0733.

IN THE CLAIMS

Please substitute the following set of claims for those currently pending.

1. (Original) A method for detecting a cancer-associated mutant nucleic acid that is present in a patient sample at a low level relative to a corresponding wild-type nucleic acid, the method comprising:

diluting nucleic acids in a biological sample to form a set comprising a plurality of assay samples;

amplifying the nucleic acids in the assay samples to form a population of amplified molecules;

performing an assay on the amplified molecules in each assay sample to determine whether a cancer-associated mutation is present in at least one of the assay samples;

wherein the step of diluting in performed until at least one-fiftieth of the assay samples in the set comprise a number (N) of molecules such that 1/N is larger than a ratio of the mutant nucleic acid to the wild-type nucleic acid required to detect the mutant nucleic acid if it is present in the assay sample.

2. (Original) The method of claim 1 wherein the step of diluting is performed until between 0.1 and 0.9 of the assay samples yield an amplification product when subjected to a polymerase chain reaction.
3. (Original) The method of claim 1 wherein the step of diluting is performed until all of the assay samples yield an amplification product when subjected to a polymerase chain reaction and each assay sample contains less than 10 nucleic acid template molecules containing a reference genetic sequence.
4. (Original) The method of claim 1 wherein the step of diluting is performed until all of the assay samples yield an amplification product when subjected to a polymerase chain reaction and each assay sample contains less than 100 nucleic acid template molecules containing a reference genetic sequence.
5. (Original) The method of claim 1 wherein the biological sample is cell-free.
6. (Original) The method of claim 1 wherein the number of assay samples within the set is greater than 10.

7. (Original) The method of claim 1 wherein the number of assay samples within the set is greater than 50.
8. (Original) The method of claim 1 wherein the number of assay samples within the set is greater than 100.
9. (Original) The method of claim 1 wherein the number of assay samples within the set is greater than 500.
10. (Original) The method of claim 1 wherein the number of assay samples within the set is greater than 1000.
11. (Original) The method of claim 1 wherein the step of amplifying and the step of analyzing are performed on assay samples in the same receptacle.
12. (Original) The method of claim 1 wherein a molecular beacon probe is used in the step of analyzing, wherein a molecular beacon probe is an oligonucleotide with a stem-loop structure having a photoluminescent dye at one of the 5' or 3' ends and a quenching agent at the opposite 5' or 3' end.
13. (Original) The method of claim 1 wherein the step of analyzing employs gel electrophoresis.
14. (Original) The method of claim 1 wherein the step of analyzing employs hybridization to at least one nucleic acid probe.
15. (Original) The method of claim 1 wherein the step of analyzing employs hybridization to at least two nucleic acid probe.
16. (Original) The method of claim 12 wherein two molecular beacon probes are used, each having a different photoluminescent dye.
17. (Original) The method of claim 12 wherein the molecular beacon probe detects a wild-type nucleic acid better than a mutant nucleic acid.
18. (Original) The method of claim 1 wherein the step of amplifying employs a single pair of primers.
19. (Original) The method of claim 1 wherein the step of amplifying employs a polymerase which is activated only after heating.
20. (Original) The method of claim 1 wherein the step of amplifying employs at least 40 cycles of heating and cooling.

21. (Original) The method of claim 1 wherein the step of amplifying employs at least 50 cycles of heating and cooling.

22. (Original) The method of claim 1 wherein the step of amplifying employs at least 60 cycles of heating and cooling.

23. (Original) The method of claim 1 wherein the biological sample is selected from the group consisting of stool, blood, and lymph nodes.

24. (Original) The method of claim 1 wherein the biological sample is blood or bone marrow of a leukemia or lymphoma patient who has received anti-cancer therapy.

25. (Original) The method of claim 1 wherein the mutant nucleic acid is a translocated allele

26. (Original) The method of claim 1 wherein the mutant nucleic acid is within an amplicon which is amplified during neoplastic development.

27. (Original) The method of claim 1 wherein the mutant nucleic acid is a rare exon sequence

28. (Original) The method of claim 1 wherein the nucleic acids being analyzed comprise cDNA of RNA transcripts.

29. (Original) A method for determining the ratio of a selected genetic sequence in a population of genetic sequences from a **blood** sample, comprising the steps of:

diluting nucleic acid template molecules from a **blood** sample to form a set comprising a plurality of assay samples;

amplifying the template molecules within the assay samples to form a population of amplified molecules in the assay samples of the set;

analyzing the amplified molecules in the assay samples of the set to determine a first number of assay samples which contain the selected genetic sequence and a second number of assay samples which contain a reference genetic sequence;

comparing the first number to the second number to ascertain a ratio which reflects the composition of the **blood** sample.

30. (Original) The method of claim 29 wherein the step of amplifying employs **real-time polymerase chain reactions**.

31. (Original) The method of claim 30 wherein the real-time polymerase chain reactions comprise a **dual-labeled fluorogenic probe**.

32. (Original) The method of claim 29 further comprising the step of :
identifying **an allelic imbalance** based on the ratio ascertained.

33. (Original) The method of claim 29 wherein the selected genetic sequences and the reference genetic sequence are **non-polymorphic markers**.

34. (Original) The method of claim 29 wherein the selected genetic sequence and the reference genetic sequence are **on distinct chromosomes**.

35. (Original) A method for determining the ratio of a selected **non-polymorphic marker** in a population of genetic sequences in a **biological** sample, comprising the steps of:

diluting nucleic acid template molecules in a biological sample to form a set comprising a plurality of assay samples;

amplifying the template molecules within the assay samples to form a population of amplified molecules in the assay samples of the set;

analyzing the amplified molecules in the assay samples of the set to determine a first number of assay samples which contain the selected **non-polymorphic marker** and a second number of assay samples which contain a reference **non-polymorphic marker, wherein the selected and reference non-polymorphic markers are on distinct chromosomes**;

comparing the first number to the second number to ascertain a ratio which reflects the composition of the **biological** sample; and

identifying **an allelic imbalance** based on the ratio ascertained.

36. (Original) The method of claim 35 wherein the biological sample is a blood sample.

37. (Original) The method of claim 35 wherein the step of amplifying employs real-time polymerase chain reactions.

38. (Original) The method of claim 37 wherein the real-time polymerase chain reactions comprise a dual-labeled fluorogenic probe.

39. (Original) A method for determining the ratio of a selected genetic sequence in a population of genetic sequences from a **blood** sample, comprising the steps of:

amplifying template molecules within a set comprising a plurality of assay samples to form a population of amplified molecules in each of the assay samples of the set, wherein the template molecules are obtained from a **blood** sample;

analyzing the amplified molecules in the assay samples of the set to determine a first number of assay samples which contain the selected genetic sequence and a second number of assay samples which contain a reference genetic sequence, wherein at least one-fiftieth of the assay samples in the set comprise a number (N) of molecules such that 1/N is larger than the ratio of selected genetic sequences to total genetic sequences required to determine the presence of the selected genetic sequence;

comparing the first number to the second number to ascertain a ratio which reflects the composition of the **blood** sample.

40. (Original) The method of claim 39 wherein the step of amplifying employs **real-time polymerase chain reactions**.

41. (Original) The method of claim 40 wherein the real-time polymerase chain reactions comprise a **dual-labeled fluorogenic probe**.

42. (Original) The method of claim 39 further comprising the step of :

identifying **an allelic imbalance** based on the ratio ascertained.

43. (Original) The method of claim 39 wherein the selected genetic sequences and the reference genetic sequence are **non-polymorphic markers**.

44. (Original) The method of claim 39 wherein the selected genetic sequence and the reference genetic sequence are **on distinct chromosomes**.

45. (Original) A method for determining the ratio of a selected **non-polymorphic marker** in a population of **non-polymorphic markers** from a **biological** sample, comprising the steps of:

amplifying template molecules within a set comprising a plurality of assay samples to form a population of amplified molecules in each of the assay samples of the set , wherein the template molecules are obtained from a biological sample;

analyzing the amplified molecules in the assay samples of the set to determine a first number of assay samples which contain the selected **non-polymorphic marker** and a second number of assay samples which contain a reference **non-polymorphic marker**, wherein at least one-fiftieth of the assay samples in the set comprise a number (N) of molecules such that 1/N is larger than the ratio of selected **non-polymorphic marker** to total **non-polymorphic markers** required to determine the presence of the selected **non-polymorphic marker,** wherein the selected genetic sequence and the reference genetic sequence are **on distinct chromosomes**;

comparing the first number to the second number to ascertain a ratio which reflects the composition of the biological sample; and

identifying **an allelic imbalance based on the ratio ascertained.**

46. (Original) The method of claim 45 wherein the step of amplifying employs real-time polymerase chain reactions.

47. (Original) The method of claim 46 wherein the real-time polymerase chain reactions comprise a dual-labeled fluorogenic probe.

48. (Original) The method of claim 45 wherein the biological sample is from blood.

49. (New) A method for detecting a genetic sequence in a mixed population of nucleic acid sequences, comprising:

distributing or diluting a mixed population of nucleic acid sequences into at least ten assay samples such that said at least ten assay samples each comprises less than ten template molecules;

amplifying the template molecules in the assay samples, wherein an assay sample with a single template molecule forms homogeneous amplification products in the assay sample;

determining nucleic acid sequence of amplification products from an assay sample with homogeneous amplification products.

50. (New) The method of claim 1 wherein each of the assay samples has on average 0.5 molecules of template.

51. (New) The method of claim 1 wherein between 0.1 and 0.9 of the assay samples yield an amplification product.

52. (New) The method of claim 1 wherein the mixed population of nucleic acid sequences is distributed or diluted to a single template molecule level in the assay samples.

53. (New) The method of claim 1 wherein the mixed population of nucleic acid sequences is from a tissue or body sample.

54. (New) The method of claim 1 wherein the mixed population of nucleic acids sequences is from a sample selected from the group consisting of stool, blood, and lymph nodes.

55. (New) The method of claim 1 wherein the mixed population of nucleic acids sequences is distributed or diluted such that at least fifteen assay samples comprise less than ten template molecules.

56. (New) The method of claim 1 wherein the mixed population of nucleic acids sequences is distributed or diluted such that at least twenty assay samples comprise less than ten template molecules.

57. (New) The method of claim 1 wherein the mixed population of nucleic acids sequences is distributed or diluted such that at least twenty-five assay samples comprise less than ten template molecules.
58. (New) The method of claim 1 wherein the mixed population of nucleic acids sequences is distributed or diluted such that at least thirty assay samples comprise less than ten template molecules.
59. (New) The method of claim 1 wherein the mixed population of nucleic acids sequences is distributed or diluted such that at least forty assay samples comprise less than ten template molecules.
60. (New) The method of claim 1 wherein the mixed population of nucleic acids sequences is distributed or diluted such that at least fifty assay samples comprise less than ten template molecules.
61. (New) The method of claim 1 wherein the mixed population of nucleic acids sequences is distributed or diluted such that at least seventy-five assay samples comprise less than ten template molecules.
62. (New) The method of claim 1 wherein the mixed population of nucleic acids sequences is distributed or diluted such that at least one hundred assay samples comprise less than ten template molecules.
63. (New) The method of claim 1 wherein the mixed population of nucleic acids sequences is distributed or diluted such that at least five hundred assay samples comprise less than ten template molecules.
64. (New) The method of claim 1 wherein the mixed population of nucleic acids sequences is distributed or diluted such that at least one thousand assay samples comprise less than ten template molecules.
65. (New) The method of claim 1 wherein the mixed population of nucleic acids sequences is distributed or diluted such that at least one thousand assay samples are diluted to a single template molecule level.
66. (New) The method of claim 1 wherein the mixed population of nucleic acids sequences is distributed or diluted such that at least one thousand assay samples has on average 0.5 molecules of template.

67. (New) The method of claim 1 wherein the mixed population of nucleic acids sequences is distributed or diluted such that between 0.1 and 0.9 of at least one thousand assay samples yield an amplification product.

68. (New) The method of claim 1 wherein the mixed population of nucleic acids sequences is distributed or diluted such that one half of at least one thousand assay samples have one template molecule.

Remarks

The new claims are fully supported in the application as filed, as indicated below.

Claim	Claim recitations	Specification Support
49	A method for detecting a genetic sequence in a mixed population of nucleic acid sequences, comprising:	Thus there is a need in the art for methods for accurately and quantitatively detecting genetic sequences in mixed populations of sequences. Page 2, second full paragraph
	distributing or diluting a mixed population of nucleic acid sequences into at least ten assay samples such that said at least ten assay samples each comprises less than ten template molecules;	The biological sample is diluted to a point at which a practically usable number of the diluted samples contain a proportion of the selected genetic sequence (analyte) relative to total template molecules such that the analyzing technique being used can detect the analyte…. Alternatively, dilute sources of template nucleic acids can be used. Page 7, paragraph 2 Preferably at least ten diluted assay samples are amplified and analyzed. Page 10, paragraph 3 Desirably each assay sample prior to amplification will contain less than a hundred or less than ten template molecules. Page 7, paragraph 2
	amplifying the template molecules in the assay samples, wherein an assay sample with a single template molecule forms homogeneous amplification products in the assay sample;	At its limit, single template molecules can be amplified so that the products are completely mutant or completely wild-type

Claim	Claim recitations	Specification Support
		(WT). The homogeneity of these amplification products makes them trivial to distinguish through existing techniques. Page 6, last paragraph.
	determining nucleic acid sequence of amplification products from an assay sample with homogeneous amplification products.	Determining the sequence of the putative mutants in the positive wells, by direct sequencing as performed here or by any of the other techniques….Page 8, first paragraph Although the working examples demonstrate the use of molecular beacon probes as the means of analysis of the amplified dilution samples, other techniques can be used as well. These include sequencing….Page 12, second full paragraph
50	The method of claim 1 wherein each of the assay samples has on average 0.5 molecules of template.	A particularly preferred degree of dilution is to a point where each of the assay samples has on average one-half of a template. Page 7, paragraph 2
51	The method of claim 1 wherein between 0.1 and 0.9 of the assay samples yield an amplification product	To achieve a dilution to approximately a single template molecule level, one can dilute such that between 0.1 and 0.9 of the assay samples yield an amplification product. Page 10, paragraph 2
52	The method of claim 1 wherein the mixed population of nucleic acid sequences is distributed or diluted to a single template molecule level in the assay samples.	To achieve a dilution to approximately a single template molecule level, one can dilute such that between 0.1 and 0.9 of the assay samples yield an amplification product. Page 10, paragraph 2
53	The method of claim 1 wherein the mixed population of nucleic acid sequences is from a	Biological samples which can be used as the starting material for the analyses may be from any

Claim	Claim recitations	Specification Support
	tissue or body sample.	tissue or body sample from which DNA or mRNA can be isolated. Page 11, first full paragraph
54	The method of claim 1 wherein the mixed population of nucleic acids sequences is from a sample selected from the group consisting of stool, blood, and lymph nodes.	Preferred sources include stool, blood, and lymph nodes. Page 11, first full paragraph
55	The method of claim 1 wherein the mixed population of nucleic acids sequences is distributed or diluted such that at least fifteen assay samples comprise less than ten template molecules.	Preferably at least ten diluted assay samples are amplified and analyzed. More preferably at least 15, 20, 25, 30, 40, 50, 75, 100, 500, or 1000 diluted assay samples are amplified and analyzed. Page 10, paragraph 3 Desirably each assay sample prior to amplification will contain less than a hundred or less than ten template molecules. Page 7, paragraph 2
56	The method of claim 1 wherein the mixed population of nucleic acids sequences is distributed or diluted such that at least twenty assay samples comprise less than ten template molecules.	Preferably at least ten diluted assay samples are amplified and analyzed. More preferably at least 15, 20, 25, 30, 40, 50, 75, 100, 500, or 1000 diluted assay samples are amplified and analyzed. Page 10, paragraph 3 Desirably each assay sample prior to amplification will contain less than a hundred or less than ten template molecules. Page 7, paragraph 2
57	The method of claim 1 wherein the mixed population of nucleic acids sequences is distributed or diluted such that at least twenty-five assay samples comprise less than ten template molecules.	Preferably at least ten diluted assay samples are amplified and analyzed. More preferably at least 15, 20, 25, 30, 40, 50, 75, 100, 500, or 1000 diluted assay samples are amplified and analyzed. Page 10, paragraph 3 Desirably each assay sample prior to amplification will

Claim	Claim recitations	Specification Support
		contain less than a hundred or less than ten template molecules. Page 7, paragraph 2
58	The method of claim 1 wherein the mixed population of nucleic acids sequences is distributed or diluted such that at least thirty assay samples comprise less than ten template molecules.	Preferably at least ten diluted assay samples are amplified and analyzed. More preferably at least 15, 20, 25, 30, 40, 50, 75, 100, 500, or 1000 diluted assay samples are amplified and analyzed. Page 10, paragraph 3 Desirably each assay sample prior to amplification will contain less than a hundred or less than ten template molecules. Page 7, paragraph 2
59	The method of claim 1 wherein the mixed population of nucleic acids sequences is distributed or diluted such that at least forty assay samples comprise less than ten template molecules.	Preferably at least ten diluted assay samples are amplified and analyzed. More preferably at least 15, 20, 25, 30, 40, 50, 75, 100, 500, or 1000 diluted assay samples are amplified and analyzed. Page 10, paragraph 3 Desirably each assay sample prior to amplification will contain less than a hundred or less than ten template molecules. Page 7, paragraph 2
60	The method of claim 1 wherein the mixed population of nucleic acids sequences is distributed or diluted such that at least fifty assay samples comprise less than ten template molecules.	Preferably at least ten diluted assay samples are amplified and analyzed. More preferably at least 15, 20, 25, 30, 40, 50, 75, 100, 500, or 1000 diluted assay samples are amplified and analyzed. Page 10, paragraph 3 Desirably each assay sample prior to amplification will contain less than a hundred or less than ten template molecules. Page 7, paragraph 2
61	The method of claim 1 wherein the mixed population of nucleic acids sequences is distributed or diluted such that at least seventy-	Preferably at least ten diluted assay samples are amplified and analyzed. More preferably at least 15, 20, 25, 30, 40, 50, 75,

Claim	Claim recitations	Specification Support
	five assay samples comprise less than ten template molecules.	100, 500, or 1000 diluted assay samples are amplified and analyzed. Page 10, paragraph 3 Desirably each assay sample prior to amplification will contain less than a hundred or less than ten template molecules. Page 7, paragraph 2
62	The method of claim 1 wherein the mixed population of nucleic acids sequences is distributed or diluted such that at least one hundred assay samples comprise less than ten template molecules. .	Preferably at least ten diluted assay samples are amplified and analyzed. More preferably at least 15, 20, 25, 30, 40, 50, 75, 100, 500, or 1000 diluted assay samples are amplified and analyzed. Page 10, paragraph 3 Desirably each assay sample prior to amplification will contain less than a hundred or less than ten template molecules. Page 7, paragraph 2
63	The method of claim 1 wherein the mixed population of nucleic acids sequences is distributed or diluted such that at least five hundred assay samples comprise less than ten template molecules.	Preferably at least ten diluted assay samples are amplified and analyzed. More preferably at least 15, 20, 25, 30, 40, 50, 75, 100, 500, or 1000 diluted assay samples are amplified and analyzed. Page 10, paragraph 3 Desirably each assay sample prior to amplification will contain less than a hundred or less than ten template molecules. Page 7, paragraph 2
64	The method of claim 1 wherein the mixed population of nucleic acids sequences is distributed or diluted such that at least one thousand assay samples comprise less than ten template molecules.	Preferably at least ten diluted assay samples are amplified and analyzed. More preferably at least 15, 20, 25, 30, 40, 50, 75, 100, 500, or 1000 diluted assay samples are amplified and analyzed. Page 10, paragraph 3 Desirably each assay sample prior to amplification will contain less than a hundred or

Claim	Claim recitations	Specification Support
		less than ten template molecules. Page 7, paragraph 2
65	The method of claim 1 wherein the mixed population of nucleic acids sequences is distributed or diluted such that at least one thousand assay samples are diluted to a single template molecule level.	Preferably at least ten diluted assay samples are amplified and analyzed. More preferably at least 15, 20, 25, 30, 40, 50, 75, 100, 500, or 1000 diluted assay samples are amplified and analyzed. Page 10, paragraph 3 To achieve a dilution to approximately a single template molecule level, one can dilute such that between 0.1 and 0.9 of the assay samples yield an amplification product. Page 10, paragraph 2
66	The method of claim 1 wherein the mixed population of nucleic acids sequences is distributed or diluted such that at least one thousand assay samples has on average 0.5 molecules of template.	Preferably at least ten diluted assay samples are amplified and analyzed. More preferably at least 15, 20, 25, 30, 40, 50, 75, 100, 500, or 1000 diluted assay samples are amplified and analyzed. Page 10, paragraph 3 A particularly preferred degree of dilution is to a point where each of the assay samples has on average one-half of a template. Page 7, paragraph 2
67	The method of claim 1 wherein the mixed population of nucleic acids sequences is distributed or diluted such that between 0.1 and 0.9 of at least one thousand assay samples yield an amplification product.	Preferably at least ten diluted assay samples are amplified and analyzed. More preferably at least 15, 20, 25, 30, 40, 50, 75, 100, 500, or 1000 diluted assay samples are amplified and analyzed. Page 10, paragraph 3 To achieve a dilution to

Claim	Claim recitations	Specification Support
		approximately a single template molecule level, one can dilute such that between 0.1 and 0.9 of the assay samples yield an amplification product. Page 10, paragraph 2
68	The method of claim 1 wherein the mixed population of nucleic acids sequences is distributed or diluted such that one half of at least one thousand assay samples have at least one template molecule.	Preferably at least ten diluted assay samples are amplified and analyzed. More preferably at least 15, 20, 25, 30, 40, 50, 75, 100, 500, or 1000 diluted assay samples are amplified and analyzed. Page 10, paragraph 3 In one preferred embodiment each diluted sample has on average one half a template molecule. This is the same as one half of the diluted samples having one template molecule. Page 10, paragraph 1

Respectfully submitted,

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Dated: November 30, 2011

Electronic Patent Application Fee Transmittal

Application Number:	13071105
Filing Date:	24-Mar-2011
Title of Invention:	Digital Amplification
First Named Inventor/Applicant Name:	Bert VOGELSTEIN
Filer:	Sarah Anne Kagan.
Attorney Docket Number:	001107.00866

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:				
Claims in excess of 20	1202	20	60	1200
Independent claims in excess of 3	1201	1	250	250
Miscellaneous-Filing:				
Petition:				
Patent-Appeals-and-Interference:				
Post-Allowance-and-Post-Issuance:				

Description	Fee Code	Quantity	Amount	Sub-Total in USD($)
Extension-of-Time:				
Miscellaneous:				
			Total in USD ($)	**1450**

Electronic Acknowledgement Receipt

EFS ID:	11509270
Application Number:	13071105
International Application Number:	
Confirmation Number:	3361
Title of Invention:	Digital Amplification
First Named Inventor/Applicant Name:	Bert VOGELSTEIN
Customer Number:	11332
Filer:	Sarah Anne Kagan.
Filer Authorized By:	
Attorney Docket Number:	001107.00866
Receipt Date:	30-NOV-2011
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Payment information:

Submitted with Payment	yes
Payment Type	Deposit Account
Payment was successfully received in RAM	$1450
RAM confirmation Number	3
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Authorized User	

File Listing:

Document Number	Document Description	File Name	File Size(Bytes)/ Message Digest	Multi Part /.zip	Pages (if appl.)

1	Preliminary Amendment	001107prelim00866amd.pdf	112622 2d05049633ecde45742e71819abd860f17ebd9f5	no	17
Warnings:					
Information:					
2	Fee Worksheet (SB06)	fee-info.pdf	31434 c6b0ee1754b1d8e29ac3e2d1306b90b2ce2fa060	no	2
Warnings:					
Information:					
			Total Files Size (in bytes):	144056	

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 (07-06)
Approved for use through 1/31/2007. OMB 0651-0032
U.S. Patent and Trademark Office; U.S. DEPARTMENT OF COMMERCE

PATENT APPLICATION FEE DETERMINATION RECORD

Substitute for Form PTO-875

Application or Docket Number	Filing Date	☐ To be Mailed
13/071,105	03/24/2011	

APPLICATION AS FILED – PART I

FOR	(Column 1) NUMBER FILED	(Column 2) NUMBER EXTRA	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	
☐ SEARCH FEE (37 CFR 1.16(k), (i), or (m))	N/A	N/A	N/A			N/A	
☐ EXAMINATION FEE (37 CFR 1.16(o), (p), or (q))	N/A	N/A	N/A			N/A	
TOTAL CLAIMS (37 CFR 1.16(i))	minus 20 =	*	X $ =		OR	X $ =	
INDEPENDENT CLAIMS (37 CFR 1.16(h))	minus 3 =	*	X $ =			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 $250 ($125 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			TOTAL	

APPLICATION AS AMENDED – PART II

AMENDMENT		(Column 1) CLAIMS REMAINING AFTER AMENDMENT		(Column 2) HIGHEST NUMBER PREVIOUSLY PAID FOR	(Column 3) PRESENT EXTRA	SMALL ENTITY RATE ($)	SMALL ENTITY ADDITIONAL FEE ($)	OR	OTHER THAN SMALL ENTITY RATE ($)	OTHER THAN SMALL ENTITY ADDITIONAL FEE ($)
	11/30/2011									
	Total (37 CFR 1.16(i))	* 68	Minus	** 48	= 20	X $ =		OR	X $60=	1200
	Independent (37 CFR 1.16(h))	* 6	Minus	***5	= 1	X $ =		OR	X $250=	250
	☐ 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	1450

AMENDMENT		(Column 1) CLAIMS REMAINING AFTER AMENDMENT		(Column 2) HIGHEST NUMBER PREVIOUSLY PAID FOR	(Column 3) PRESENT EXTRA	RATE ($)	ADDITIONAL FEE ($)		RATE ($)	ADDITIONAL FEE ($)
	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 number found in the appropriate box in column 1.

Legal Instrument Examiner:
/ELMIRA HALL/

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APPLICATION NO.	FILING DATE	FIRST NAMED INVENTOR	ATTORNEY DOCKET NO.	CONFIRMATION NO.
13/071,105	03/24/2011	Bert VOGELSTEIN	001107.00866	3361

11332 7590 04/10/2012

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EXAMINER	
WOOLWINE, SAMUEL C	
ART UNIT	PAPER NUMBER
1637	

MAIL DATE	DELIVERY MODE
04/10/2012	PAPER

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.

Office Action Summary	Application No.	Applicant(s)	
	13/071,105	VOGELSTEIN ET AL.	
	Examiner	Art Unit	
	SAMUEL WOOLWINE	1637	

-- 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 *1* MONTH(S) OR THIRTY (30) DAYS, WHICHEVER IS LONGER, 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 _____.

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) *1-68* 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) *1-68* are subject to restriction and/or election requirement.

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).

12)☐ The oath or declaration is objected to by the Examiner. Note the attached Office Action or form PTO-152.

Priority under 35 U.S.C. § 119

13)☐ Acknowledgment is made of a claim for foreign priority under 35 U.S.C. § 119(a)-(d) or (f).

a)☐ All b)☐ Some * c)☐ None of:

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) ☐ Notice of Draftsperson's Patent Drawing Review (PTO-948)
3) ☐ Information Disclosure Statement(s) (PTO/SB/08) Paper No(s)/Mail Date _____.
4) ☐ Interview Summary (PTO-413) Paper No(s)/Mail Date. _____ .
5) ☐ Notice of Informal Patent Application
6) ☐ Other: _____.

DETAILED ACTION

Note: New claims 50-68 depend from original claim 1, rather than new independent claim 49. Applicant is requested to confirm whether this is the case. The restriction requirement set forth below applies to the claims as written. If claims 50-68 were intended to depend from claim 49, those claims will be included in Group IV rather than Group I.

Election/Restrictions

Restriction to one of the following inventions is required under 35 U.S.C. 121:

I. Claims 1-28 and 50-68, drawn to methods for detecting a cancer-associated mutation comprising diluting until at least 1/50 of the assay samples comprise a number (N) of molecules such that 1/N is larger than a ratio of the mutant nucleic acid to the wild-type nucleic acid required to detect the mutant nucleic acid if it is present in the assay sample, classified in class 435, subclass 6.12.

II. Claims 29-38, drawn to methods for determining a ratio of a selected genetic sequence to a reference genetic sequence comprising determining a first number of assay samples which contain the selected genetic sequence and a second number of assay samples which contain a reference genetic sequence and comparing the first number to the second number to ascertain a ratio, classified in class 435, subclass 6.12.

III. Claims 39-48, drawn to methods for determining a ratio of a selected genetic sequence to a reference genetic sequence wherein at least 1/50

of the assay samples comprise a number (N) of molecules such that 1/N is larger than a ratio of selected genetic sequences to total genetic sequences required to determine the presence of the selected genetic sequence and determining a first number of assay samples which contain the selected genetic sequence and a second number of assay samples which contain a reference genetic sequence and comparing the first number to the second number to ascertain a ratio, classified in class 436, subclass 6.12.

IV. Claim 49, drawn to methods for detecting a genetic sequence comprising distributing or diluting a mixed population of nucleic acid sequences into at least 10 assay samples such that at least 10 assay samples each comprise less than 10 template molecules and determining nucleic acid sequence of amplification products from an assay sample with homogeneous amplification products, classified in class 435, subclass 6.12.

The inventions are distinct, each from the other because of the following reasons:

Inventions I and (II & III) are unrelated. Inventions are unrelated if it can be shown that they are not disclosed as capable of use together and they have different designs, modes of operation, and effects (MPEP § 802.01 and § 806.06). In the instant case, the different inventions have different designs. Invention I requires detection of a cancer-associated mutation, which is not required for inventions II & III. Inventions II & III require determining the ratio of a selected genetic sequence to a reference genetic

sequence, which is not required for invention I. In addition, invention I requires at least 1/50 of the assay samples comprise a number (N) of molecules such that 1/N is larger than a ratio of a nucleic acid comprising a cancer-associated mutation *to wild-type nucleic acid* required to determine the presence of the mutation, which is not required by invention III. Note that in invention III, what is required is that 1/50 of the assay samples comprise a number (N) of molecules such that 1/N is larger than a ratio of a selected genetic sequence *to total nucleic acid* (which is presumed, based on plain language, to mean "selected" genetic sequence + "reference" genetic sequence). This is not required for invention I.

Inventions II and III are unrelated. Inventions are unrelated if it can be shown that they are not disclosed as capable of use together and they have different designs, modes of operation, and effects (MPEP § 802.01 and § 806.06). In the instant case, the different inventions have different designs. Invention II requires diluting nucleic acid template molecules from a sample to form a plurality of assay samples, which is not required for invention III. Invention III requires at least 1/50 of the assay samples comprise a number (N) of molecules such that 1/N is larger than a ratio of selected genetic sequences to total genetic sequences required to determine the presence of the selected genetic sequence, which is not required for invention II.

Inventions IV and (I-III) are unrelated. Inventions are unrelated if it can be shown that they are not disclosed as capable of use together and they have different designs, modes of operation, and effects (MPEP § 802.01 and § 806.06). In the instant case, the different inventions have different designs. Invention IV requires distributing or diluting

a mixed population of nucleic acid sequences into at least ten assay samples such that at least ten assay samples each comprise less than ten template molecules, which is not required by inventions I-III. Inventions II & III require determining a first number of assay samples which contain the selected genetic sequence and a second number of assay samples which contain a reference genetic sequence and comparing the first number to the second number to ascertain a ratio, which is not required by invention IV. In addition, invention I requires at least 1/50 of the assay samples comprise a number (N) of molecules such that 1/N is larger than a ratio of a nucleic acid comprising a cancer-associated mutation to wild-type nucleic acid required to determine the presence of the mutation, which is not required by invention IV. Invention III requires at least 1/50 of the assay samples comprise a number (N) of molecules such that 1/N is larger than a ratio of selected genetic sequences to total genetic sequences required to determine the presence of the selected genetic sequence, which is not required for invention IV.

Restriction for examination purposes as indicated is proper because all these inventions listed in this action are independent or distinct for the reasons given above and there would be a serious search and/or examination burden if restriction were not required because at least the following reason(s) apply:

Due to the fact that each group of inventions requires limitations not required by the other groups, a search each invention would be conducted using different search strategies. While these different searches might be partially overlapping, they would not be the same. This would place an undue burden of search on the Office.

Applicant is advised that the reply to this requirement to be complete must include (i) an election of a invention to be examined even though the requirement may be traversed (37 CFR 1.143) **and (ii) identification of the claims encompassing the elected invention**.

The election of an invention may be made with or without traverse. To reserve 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 restriction 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 upon the elected invention.

Should applicant traverse on the ground that the inventions are not patentably distinct, applicant should submit evidence or identify such evidence now of record showing the inventions 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 inventions 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 invention.

This application contains claims directed to the following patentably distinct species:

A1: less than all assay samples yield an amplification product/contain template (claims 2, 50, 51, 66, 67).

A2: all assay samples yield an amplification product/contain template (claims 3, 4, 52, 65).

B1: analyzing performed in same receptacle/employs hybridization probes (claims 11, 12, 14-17, 31, 38, 41, 47).

B2: analyzing employs gel electrophoresis (claim 13).

C1: sample is stool (claims 23 in-part, 54 in-part).

C2: sample is blood (claims 23 in-part, 24 in-part, 29-34, 36, 39-44, 48, 54 in-part).

C3: sample is lymph node (claims 23 in-part, 54 in-part).

C4: sample is bone marrow (claim 24 in-part).

D1: mutation is translocated allele (claim 25).

D2: mutation is a nucleic acid sequence amplified during neoplastic development (claim 26).

D3: mutation is a rare exon sequence (claim 27).

The species are independent or distinct because each species within a group A, B, C or D is mutually exclusive of the others. In addition, these species are not obvious variants of each other based on the current record.

Applicant is required under 35 U.S.C. 121 to elect a single disclosed species from each of A, B, C and D for prosecution on the merits to which the claims shall be restricted if no generic claim is finally held to be allowable. Depending on the *invention* elected (I-IV), some elections of species may not apply. Currently, claims 1, 5-10, 18-22, 28, 35, 37, 45, 46, 49, 53, 55-64 and 68 are generic.

There is a search and/or examination burden for the patentably distinct species as set forth above because at least the following reason(s) apply:

Since each species within a group A, B, C or D is mutually exclusive of the others and would require a different search. There is no certainty that a prior art reference anticipating or rendering obvious any one species would be applicable to the others.

Applicant is advised that the reply to this requirement to be complete must include (i) an election of a species or a grouping of patentably indistinct 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 or grouping of patentably indistinct 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.

The election 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.

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.

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).

Any inquiry concerning this communication or earlier communications from the examiner should be directed to SAMUEL WOOLWINE whose telephone number is (571)272-1144. The examiner can normally be reached on Mon-Fri 9:00am-5:00pm.

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.

/Samuel Woolwine/
Primary Examiner

PATENT

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re Application of	)	
	)	
Bert VOGELSTEIN et al.	)	Examiner: WOOLWINE, Samuel C.
	)	
Serial No. 13/071,105	)	
	)	Group Art Unit: 1637
	)	
Filed: March 24, 2011	)	Confirmation No. 3361
	)	
For: **Digital Amplification**	)	Atty. Dkt. No. 001107.00866

RESPONSE TO RESTRICTION REQUIREMENT

Commissioner of Patents
P.O. Box 1450
Alexandria, VA 22313-1450

Sir:

In response to the office action mailed May 10, 2012, applicants elect claim Group IV (claim 49 and 50-68, drawn to methods for detecting a generic sequence comprising distributing or diluting a mixed population of nucleic acid sequences into at least 10 assay samples such that at least 10 assay samples each comprise less than 10 template molecules and determining nucleic acid sequence of amplification products from an assay sample with homogeneous amplification products). Claims 50-68 are amended below to depend from claim 49.

In addition, applicants elect species: A-1 (less than all), B-1 (in same pot), C-2 (blood), and D-3 (a rare exon sequence). Claims which read on all of the elected species are: claims 49-68.

The Commissioner is authorized to charge any fees which may be required or credit any overpayment to our Deposit Account 19-0733.

IN THE CLAIMS

Please replace the following claim set for that currently of record.

1. (Original) A method for detecting a cancer-associated mutant nucleic acid that is present in a patient sample at a low level relative to a corresponding wild-type nucleic acid, the method comprising:

diluting nucleic acids in a biological sample to form a set comprising a plurality of assay samples;

amplifying the nucleic acids in the assay samples to form a population of amplified molecules;

performing an assay on the amplified molecules in each assay sample to determine whether a cancer-associated mutation is present in at least one of the assay samples;

wherein the step of diluting in performed until at least one-fiftieth of the assay samples in the set comprise a number (N) of molecules such that 1/N is larger than a ratio of the mutant nucleic acid to the wild-type nucleic acid required to detect the mutant nucleic acid if it is present in the assay sample.

2. (Original) The method of claim 1 wherein the step of diluting is performed until between 0.1 and 0.9 of the assay samples yield an amplification product when subjected to a polymerase chain reaction.

3. (Original) The method of claim 1 wherein the step of diluting is performed until all of the assay samples yield an amplification product when subjected to a polymerase chain reaction and each assay sample contains less than 10 nucleic acid template molecules containing a reference genetic sequence.

4. (Original) The method of claim 1 wherein the step of diluting is performed until all of the assay samples yield an amplification product when subjected to a polymerase chain reaction and each assay sample contains less than 100 nucleic acid template molecules containing a reference genetic sequence.

5. (Original) The method of claim 1 wherein the biological sample is cell-free.

6. (Original) The method of claim 1 wherein the number of assay samples within the set is greater than 10.

7. (Original) The method of claim 1 wherein the number of assay samples within the set is greater than 50.

8. (Original) The method of claim 1 wherein the number of assay samples within the set is greater than 100.

9. (Original) The method of claim 1 wherein the number of assay samples within the set is greater than 500.

10. (Original) The method of claim 1 wherein the number of assay samples within the set is greater than 1000.

11. (Original) The method of claim 1 wherein the step of amplifying and the step of analyzing are performed on assay samples in the same receptacle.

12. (Original) The method of claim 1 wherein a molecular beacon probe is used in the step of analyzing, wherein a molecular beacon probe is an oligonucleotide with a stem-loop structure having a photoluminescent dye at one of the 5' or 3' ends and a quenching agent at the opposite 5' or 3' end.

13. (Original) The method of claim 1 wherein the step of analyzing employs gel electrophoresis.

14. (Original) The method of claim 1 wherein the step of analyzing employs hybridization to at least one nucleic acid probe.

15. (Original) The method of claim 1 wherein the step of analyzing employs hybridization to at least two nucleic acid probe.

16. (Original) The method of claim 12 wherein two molecular beacon probes are used, each having a different photoluminescent dye.

17. (Original) The method of claim 12 wherein the molecular beacon probe detects a wild-type nucleic acid better than a mutant nucleic acid.

18. (Original) The method of claim 1 wherein the step of amplifying employs a single pair of primers.

19. (Original) The method of claim 1 wherein the step of amplifying employs a polymerase which is activated only after heating.

20. (Original) The method of claim 1 wherein the step of amplifying employs at least 40 cycles of heating and cooling.

21. (Original) The method of claim 1 wherein the step of amplifying employs at least 50 cycles of heating and cooling.

22. (Original) The method of claim 1 wherein the step of amplifying employs at least 60 cycles of heating and cooling.

23. (Original) The method of claim 1 wherein the biological sample is selected from the group consisting of stool, blood, and lymph nodes.

24. (Original) The method of claim 1 wherein the biological sample is blood or bone marrow of a leukemia or lymphoma patient who has received anti-cancer therapy.

25. (Original) The method of claim 1 wherein the mutant nucleic acid is a translocated allele

26. (Original) The method of claim 1 wherein the mutant nucleic acid is within an amplicon which is amplified during neoplastic development.

27. (Currently amended) The method of claim 1 wherein the mutant nucleic acid is a rare exon sequence.

28. (Original) The method of claim 1 wherein the nucleic acids being analyzed comprise cDNA of RNA transcripts.

29. (Original) A method for determining the ratio of a selected genetic sequence in a population of genetic sequences from a blood sample, comprising the steps of:

diluting nucleic acid template molecules from a blood sample to form a set comprising a plurality of assay samples;

amplifying the template molecules within the assay samples to form a population of amplified molecules in the assay samples of the set;

analyzing the amplified molecules in the assay samples of the set to determine a first number of assay samples which contain the selected genetic sequence and a second number of assay samples which contain a reference genetic sequence;

comparing the first number to the second number to ascertain a ratio which reflects the composition of the blood sample.

30. (Original) The method of claim 29 wherein the step of amplifying employs real-time polymerase chain reactions.

31. (Original) The method of claim 30 wherein the real-time polymerase chain reactions comprise a dual-labeled fluorogenic probe.

32. (Original) The method of claim 29 further comprising the step of:

identifying an allelic imbalance based on the ratio ascertained.

33. (Original) The method of claim 29 wherein the selected genetic sequences and the reference genetic sequence are non-polymorphic markers.

34. (Original) The method of claim 29 wherein the selected genetic sequence and the reference genetic sequence are on distinct chromosomes.

35. (Original) A method for determining the ratio of a selected non-polymorphic marker in a population of genetic sequences in a biological sample, comprising the steps of:

diluting nucleic acid template molecules in a biological sample to form a set comprising a plurality of assay samples;

amplifying the template molecules within the assay samples to form a population of amplified molecules in the assay samples of the set;

analyzing the amplified molecules in the assay samples of the set to determine a first number of assay samples which contain the selected non-polymorphic marker and a second number of assay samples which contain a reference non-polymorphic marker, wherein the selected and reference non-polymorphic markers are on distinct chromosomes;

comparing the first number to the second number to ascertain a ratio which reflects the composition of the biological sample; and

identifying an allelic imbalance based on the ratio ascertained.

36. (Original) The method of claim 35 wherein the biological sample is a blood sample.

37. (Original) The method of claim 35 wherein the step of amplifying employs real-time polymerase chain reactions.

38. (Original) The method of claim 37 wherein the real-time polymerase chain reactions comprise a dual-labeled fluorogenic probe.

39. (Original) A method for determining the ratio of a selected genetic sequence in a population of genetic sequences from a blood sample, comprising the steps of:

amplifying template molecules within a set comprising a plurality of assay samples to form a population of amplified molecules in each of the assay samples of the set, wherein the template molecules are obtained from a blood sample;

analyzing the amplified molecules in the assay samples of the set to determine a first number of assay samples which contain the selected genetic sequence and a second number of assay samples which contain a reference genetic sequence, wherein at least one-fiftieth of the assay samples in the set comprise a number (N) of molecules such that 1/N is larger than the ratio of selected genetic sequences to total genetic sequences required to determine the presence of the selected genetic sequence;

comparing the first number to the second number to ascertain a ratio which reflects the composition of the blood sample.

40. (Original) The method of claim 39 wherein the step of amplifying employs real-time polymerase chain reactions.

41. (Original) The method of claim 40 wherein the real-time polymerase chain reactions comprise a dual-labeled fluorogenic probe.

42. (Original) The method of claim 39 further comprising the step of :

identifying an allelic imbalance based on the ratio ascertained.

43. (Original) The method of claim 39 wherein the selected genetic sequences and the reference genetic sequence are non-polymorphic markers.

44. (Original) The method of claim 39 wherein the selected genetic sequence and the reference genetic sequence are on distinct chromosomes.

45. (Original) A method for determining the ratio of a selected non-polymorphic marker in a population of non-polymorphic markers from a biological sample, comprising the steps of:

amplifying template molecules within a set comprising a plurality of assay samples to form a population of amplified molecules in each of the assay samples of the set , wherein the template molecules are obtained from a biological sample;

analyzing the amplified molecules in the assay samples of the set to determine a first number of assay samples which contain the selected non-polymorphic marker and a second number of assay samples which contain a reference non-polymorphic marker, wherein at least one-fiftieth of the assay samples in the set comprise a number (N) of molecules such that 1/N is larger than the ratio of selected non-polymorphic marker to total non-polymorphic markers required to determine the presence of the selected non-polymorphic marker, wherein the selected genetic sequence and the reference genetic sequence are on distinct chromosomes;

comparing the first number to the second number to ascertain a ratio which reflects the composition of the biological sample; and

identifying an allelic imbalance based on the ratio ascertained.

46. (Original) The method of claim 45 wherein the step of amplifying employs real-time polymerase chain reactions.

47. (Original) The method of claim 46 wherein the real-time polymerase chain reactions comprise a dual-labeled fluorogenic probe.

48. (Original) The method of claim 45 wherein the biological sample is from blood.

49. (Previously Presented) A method for detecting a genetic sequence in a mixed population of nucleic acid sequences, comprising:

distributing or diluting a mixed population of nucleic acid sequences into at least ten assay samples such that said at least ten assay samples each comprises less than ten template molecules;

amplifying the template molecules in the assay samples, wherein an assay sample with a single template molecule forms homogeneous amplification products in the assay sample;

determining nucleic acid sequence of amplification products from an assay sample with homogeneous amplification products.

50. (Currently amended) The method of claim ~~1~~ 49 wherein each of the assay samples has on average 0.5 molecules of template.

51. (Currently amended) The method of claim ~~1~~ 49 wherein between 0.1 and 0.9 of the assay samples yield an amplification product.

52. (Currently amended) The method of claim ~~1~~ 49 wherein the mixed population of nucleic acid sequences is distributed or diluted to a single template molecule level in the assay samples.

53. (Currently amended) The method of claim ~~1~~ 49 wherein the mixed population of nucleic acid sequences is from a tissue or body sample.

54. (Currently amended) The method of claim ~~1~~ 49 wherein the mixed population of nucleic acids sequences is from a sample selected from the group consisting of stool, blood, and lymph nodes.

55. (Currently amended) The method of claim ~~1~~ 49 wherein the mixed population of nucleic acids sequences is distributed or diluted such that at least fifteen assay samples comprise less than ten template molecules.

56. (Currently amended) The method of claim ~~1~~ 49 wherein the mixed population of nucleic acids sequences is distributed or diluted such that at least twenty assay samples comprise less than ten template molecules.

57. (Currently amended) The method of claim ~~1~~ 49 wherein the mixed population of nucleic acids sequences is distributed or diluted such that at least twenty-five assay samples comprise less than ten template molecules.

58. (Currently amended) The method of claim ~~1~~ 49 wherein the mixed population of nucleic acids sequences is distributed or diluted such that at least thirty assay samples comprise less than ten template molecules.

59. (Currently amended) The method of claim ~~1~~ 49 wherein the mixed population of nucleic acids sequences is distributed or diluted such that at least forty assay samples comprise less than ten template molecules.

60. (Currently amended) The method of claim ~~1~~ 49 wherein the mixed population of nucleic acids sequences is distributed or diluted such that at least fifty assay samples comprise less than ten template molecules.

61. (Currently amended) The method of claim ~~1~~ 49 wherein the mixed population of nucleic acids sequences is distributed or diluted such that at least seventy-five assay samples comprise less than ten template molecules.

62. (Currently amended) The method of claim ~~1~~ 49 wherein the mixed population of nucleic acids sequences is distributed or diluted such that at least one hundred assay samples comprise less than ten template molecules.

63. (Currently amended) The method of claim ~~1~~ 49 wherein the mixed population of nucleic acids sequences is distributed or diluted such that at least five hundred assay samples comprise less than ten template molecules.

64. (Currently amended) The method of claim ~~1~~ 49 wherein the mixed population of nucleic acids sequences is distributed or diluted such that at least one thousand assay samples comprise less than ten template molecules.

65. (Currently amended) The method of claim ~~1~~ 49 wherein the mixed population of nucleic acids sequences is distributed or diluted such that at least one thousand assay samples are diluted to a single template molecule level.

66. (Currently amended) The method of claim ~~1~~ 49 wherein the mixed population of nucleic acids sequences is distributed or diluted such that at least one thousand assay samples has on average 0.5 molecules of template.

67. (Currently amended) The method of claim ~~1~~ 49 wherein the mixed population of nucleic acids sequences is distributed or diluted such that between 0.1 and 0.9 of at least one thousand assay samples yield an amplification product.

68. (Currently amended) The method of claim ~~1~~ 49 wherein the mixed population of nucleic acids sequences is distributed or diluted such that one half of at least one thousand assay samples have one template molecule.

Respectfully submitted,

Date: June 11, 2012

By: /Sarah A. Kagan/
Sarah A. Kagan
Registration No. 32,141

Banner & Witcoff, Ltd.
Customer No. 11332

PTO/SB/22 (09-11)
Approved for use through 07/31/2012. OMB 0651-0031
U.S. Patent and Trademark Office; U.S. DEPARMENT OF COMMERCE

PETITION FOR EXTENSION OF TIME UNDER 37 CFR 1.136(a)	Docket Number (Optional) 001107.00866
Application Number 13/071,105	Filed March 24, 2011
For Digital Amplification	
Art Unit 1637	Examiner WOOLWINE, Samuel C.

This is a request under the provisions of 37 CFR 1.136(a) to extend the period for filing a reply in the above identified application.

The requested extension and fee are as follows (check time period desired and enter the appropriate fee below):

		Fee	Small Entity Fee	
☑	One month (37 CFR 1.17(a)(1))	$150	$75	$ 150.00
☐	Two months (37 CFR 1.17(a)(2))	$560	$280	$
☐	Three months (37 CFR 1.17(a)(3))	$1270	$635	$
☐	Four months (37 CFR 1.17(a)(4))	$1980	$990	$
☐	Five months (37 CFR 1.17(a)(5))	$2690	$1345	$

☐ Applicant claims small entity status. See 37 CFR 1.27.

☐ A check in the amount of the fee is enclosed.

☐ Payment by credit card. Form PTO-2038 is attached.

☐ The Director has already been authorized to charge fees in this application to a Deposit Account.

☑ The Director is hereby authorized to charge any fees which may be required, or credit any overpayment, to Deposit Account Number 190733.

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.

I am the

☐ applicant/inventor.

☐ assignee of record of the entire interest. See 37 CFR 3.71.
Statement under 37 CFR 3.73(b) is enclosed (Form PTO/SB/96).

☑ attorney or agent of record. Registration Number 32141

☐ attorney or agent under 37 CFR 1.34.
Registration number if acting under 37 CFR 1.34 _____

/Sarah A. Kagan/ — Signature

June 11, 2012 — Date

Sarah A. Kagan — Typed or printed name

(202) 824-3000 — Telephone Number

NOTE: Signatures of all the inventors or assignees of record of the entire interest or their representative(s) are required. Submit multiple forms if more than one signature is required, see below.

☑ Total of 1 forms are submitted.

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.

Electronic Patent Application Fee Transmittal

Application Number:	13071105
Filing Date:	24-Mar-2011
Title of Invention:	Digital Amplification
First Named Inventor/Applicant Name:	Bert VOGELSTEIN
Filer:	Sarah Anne Kagan./Leatrice sims
Attorney Docket Number:	001107.00866

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:				
Extension - 1 month with $0 paid	1251	1	150	150

Description	Fee Code	Quantity	Amount	Sub-Total in USD($)
Miscellaneous:				
			Total in USD ($)	**150**

Electronic Acknowledgement Receipt

EFS ID:	12978167
Application Number:	13071105
International Application Number:	
Confirmation Number:	3361
Title of Invention:	Digital Amplification
First Named Inventor/Applicant Name:	Bert VOGELSTEIN
Customer Number:	11332
Filer:	Sarah Anne Kagan./Leatrice sims
Filer Authorized By:	Sarah Anne Kagan.
Attorney Docket Number:	001107.00866
Receipt Date:	11-JUN-2012
Filing Date:	24-MAR-2011
Time Stamp:	11:37:15
Application Type:	Utility under 35 USC 111(a)

Payment information:

Submitted with Payment	yes
Payment Type	Deposit Account
Payment was successfully received in RAM	$150
RAM confirmation Number	9628
Deposit Account	190733
Authorized User	

File Listing:

Document Number	Document Description	File Name	File Size(Bytes)/ Message Digest	Multi Part /.zip	Pages (if appl.)

1		response_to_RR.pdf	83294 abfb45b23bf7dbe6e6d49bd5fb4b0d6ae1758080	yes	10

Multipart Description/PDF files in .zip description		
Document Description	**Start**	**End**
Response to Election / Restriction Filed	1	1
Claims	2	10

Warnings:

Information:

2	Extension of Time	EOT_filed_with_RR.pdf	286857 1f2225aaa12244d9c218066b5f8025f32bfb15fb	no	2

Warnings:

Information:

3	Fee Worksheet (SB06)	fee-info.pdf	30091 07c17696daf0ce93600409d2e67488efe9daf944	no	2

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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.

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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 (07-06)
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PATENT APPLICATION FEE DETERMINATION RECORD

Substitute for Form PTO-875

Application or Docket Number	Filing Date	☐ To be Mailed
13/071,105	03/24/2011	

APPLICATION AS FILED – PART I

FOR	(Column 1) NUMBER FILED	(Column 2) NUMBER EXTRA	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	
☐ SEARCH FEE (37 CFR 1.16(k), (i), or (m))	N/A	N/A	N/A			N/A	
☐ EXAMINATION FEE (37 CFR 1.16(o), (p), or (q))	N/A	N/A	N/A			N/A	
TOTAL CLAIMS (37 CFR 1.16(i))	minus 20 =	*	X $ =		OR	X $ =	
INDEPENDENT CLAIMS (37 CFR 1.16(h))	minus 3 =	*	X $ =			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 $250 ($125 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			TOTAL	

APPLICATION AS AMENDED – PART II

AMENDMENT	06/11/2012	(Column 1) CLAIMS REMAINING AFTER AMENDMENT		(Column 2) HIGHEST NUMBER PREVIOUSLY PAID FOR	(Column 3) PRESENT EXTRA	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))	* 68	Minus	** 68	= 0	X $ =		OR	X $60=	0
	Independent (37 CFR 1.16(h))	* 6	Minus	***6	= 0	X $ =		OR	X $250=	0
	☐ 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	0

AMENDMENT		(Column 1) CLAIMS REMAINING AFTER AMENDMENT		(Column 2) HIGHEST NUMBER PREVIOUSLY PAID FOR	(Column 3) PRESENT EXTRA	RATE ($)	ADDITIONAL FEE ($)		RATE ($)	ADDITIONAL FEE ($)
	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".

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Legal Instrument Examiner:
/KIM P. DOZIER/

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APPLICATION NO.	FILING DATE	FIRST NAMED INVENTOR	ATTORNEY DOCKET NO.	CONFIRMATION NO.
13/071,105	03/24/2011	Bert VOGELSTEIN	001107.00866	3361

11332 7590 10/10/2012

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EXAMINER	
WOOLWINE, SAMUEL C	
ART UNIT	PAPER NUMBER
1637	

MAIL DATE	DELIVERY MODE
10/10/2012	PAPER

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.

Office Action Summary	Application No.	Applicant(s)	
	13/071,105	VOGELSTEIN ET AL.	
	Examiner	Art Unit	
	SAMUEL WOOLWINE	1637	

-- 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* MONTH(S) OR THIRTY (30) DAYS, WHICHEVER IS LONGER, 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 *11 June 2012*.

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) *1-68* is/are pending in the application.

5a) Of the above claim(s) *1-48* is/are withdrawn from consideration.

6)☐ Claim(s) _____ is/are allowed.

7)☒ Claim(s) *49-68* is/are rejected.

8)☐ Claim(s) _____ is/are objected to.

9)☐ Claim(s) _____ are subject to restriction and/or election requirement.

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).

12)☐ The oath or declaration is objected to by the Examiner. Note the attached Office Action or form PTO-152.

Priority under 35 U.S.C. § 119

13)☐ Acknowledgment is made of a claim for foreign priority under 35 U.S.C. § 119(a)-(d) or (f).

a)☐ All b)☐ Some * c)☐ None of:

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) ☐ Notice of Draftsperson's Patent Drawing Review (PTO-948)
3) ☒ Information Disclosure Statement(s) (PTO/SB/08) Paper No(s)/Mail Date *03/24/2011*.
4) ☐ Interview Summary (PTO-413) Paper No(s)/Mail Date. _____ .
5) ☐ Notice of Informal Patent Application
6) ☐ Other: _____.

DETAILED ACTION

Election/Restrictions

Applicant's election of Group IV claims 49-68 in the reply filed on 06/11/2012 is acknowledged. Because applicant did not distinctly and specifically point out the supposed errors in the restriction requirement, the election has been treated as an election without traverse (MPEP § 818.03(a)).

Claims 1-48 are withdrawn from further consideration pursuant to 37 CFR 1.142(b) as being drawn to a nonelected invention, there being no allowable generic or linking claim. Election was made **without** traverse in the reply filed on 06/11/2012.

Claim Rejections - 35 USC § 112

The following is a quotation of the second paragraph of 35 U.S.C. 112:

> 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.

Claim 50 is rejected under 35 U.S.C. 112, second paragraph, as being indefinite for failing to particularly point out and distinctly claim the subject matter which applicant regards as the invention.

Each assay sample cannot have an *average* number of template molecules. Any sample has precisely the number of template molecules it contains. An average would be applied to a population of assay samples.

Claim Rejections - 35 USC § 102

The following is a quotation of the appropriate paragraphs of 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 –

(a) the invention was known or used by others in this country, or patented or described in a printed publication in this or a foreign country, before the invention thereof by the applicant for a patent.

(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 49, 51-53, 55-62 are rejected under 35 U.S.C. 102(b) as being anticipated by Li et al (Nature 335:414-417 (1988), cited on the IDS of 03/24/2012).

With regard to claim 49, Li taught:

distributing or diluting a mixed population of nucleic acid sequences into at least ten assay samples such that said at least ten assay samples each comprises less than ten template molecules;

The mixed population of nucleic acid sequences was "a semen sample": page 415, column 1, last [partial] paragraph: "Sperm were purified from a semen sample…". Each sperm would contain 23 chromosomes, and some sperm would comprise maternally derived chromosomes, while others would comprise paternally derived chromosomes. In addition, some sperm would comprise Y chromosomes, whereas others would comprise X chromosomes. Furthermore, the semen sample was obtained from an individual known to be heterozygous at a locus of the LDLr gene (page 415, column 1, last [full] paragraph). Li took single sperm and placed them into separate tubes for amplification (page 415, column 1, last [partial] paragraph). By doing so, Li distributed the mixed population into at least ten (in this case, 80) "assay samples" such that each of the assay samples comprised less than ten (in this case, 1) template molecules; the template, in this case, is the LDLr gene.

amplifying the template molecules in the assay samples, wherein an assay sample with a single template molecule forms homogeneous amplification products in the assay sample;

Li amplified each of the assay samples containing individual sperm; page 415, column 1, last [partial] paragraph: "Individual sperm were drawn into a fine plastic needle under microscopic observation and delivered to a tube for lysis and amplification." Since each sample containing one sperm would contain one template molecule (i.e. LDLr gene), the amplification product from such a sample would inherently be homogeneous.

determining nucleic acid sequence of amplification products from an assay sample with homogeneous amplification products.

Li amplified a known sequence: a 254 bp fragment of the LDLr gene (page 415, column 1, last [full] paragraph). The only missing sequence information was which single nucleotide polymorphism was present in each of the homogeneous amplified products, which Li determined using allele-specific oligonucleotides (see figure 2 and legend). In this way, Li determined "nucleic acid sequence of amplification products from an assay sample with homogeneous amplification products".

With regard to claim 51, Li notes that of the 80 individual sperm, 55% produced a hybridization signal (thus indicating an amplification product; page 415, sentence spanning columns 1-2). This is between 0.1 (10%) and 0.9 (90%).

With regard to claim 52, since Li distributed individual sperm to individual assay samples, he distributed to a single template molecule in the assay samples.

With regard to claim 53, semen is a "body sample" (it's a sample from a body).

With regard to claims 55-61, Li set up 80 assay samples containing less than 10 template molecules as described in the experiment on page 415 entitled "Analysis in single human sperm".

With regard to claim 62, Li performed a similar analysis on 150 individual sperm (page 415, column 2, under section entitled "Independent assortment of chromosomes".

Claims 49, 52-54 are rejected under 35 U.S.C. 102(a) as being anticipated by Irving et al (The Journal of Infectious Diseases 180:27-34, July 1999).

With regard to claims 49, 52, Irving used dilution to allow for sequence determination of individual molecules of TT virus (TTV) DNA in a sample; page 28, column 1, last [full] paragraph:

amplification of undiluted DNA extracts. Analysis of the population diversity within individual patients was done by directly sequencing the PCR products derived from amplification of multiple aliquots of DNA at the end-point dilution. Unincorporated

In one case, Irving determined the sequence of 11 different individual molecules of TTV from a single sample; page 30, column 2, last paragraph:

Analysis of viral variants within patient 30. To investigate further the possibility of viral evolution and the coexistence of distinct viral variants, multiple amplifications of single TTV DNA molecules present in the DNA extracts obtained from the first and last serum samples from patient 30 were directly sequenced. Nucleotide sequence data from 22 single molecule amplifications of samples 30a and 30e were obtained. The resulting phylogenetic analysis (figure 4) was similar but not iden-

As seen from figure 4, the "22 samples" consist of 9 molecules of TTV from the first sample, and 13 molecules of TTV from the last sample. Thus, in the latter case, there were at least 13 "assay samples" formed by dilution of the last sample, each such "assay sample" comprising a single molecule of template (which is less than 10). The assay samples were amplified, producing homogeneous products (the inherent result of amplifying a single template molecule) and sequenced. As the sequencing results indicate the original sample contained multiple genotypes of TTV, the original sample was a "mixed population of nucleic acid sequences". Not only that, the sample was serum from a human subject, which was clearly obtained in the form of blood (the true "original sample"), and as such would have also comprised all the chromosomal and mitochondrial genomic DNA, plus all the various forms of RNA (tRNA, rRNA, mRNA) present in a human blood sample. In this manner, too, it can be said that Irving began with a mixed population of nucleic acid sequences.

With regard to claims 53, 54, one of ordinary skill in the art knows that one does not obtain serum directly from a body. Rather, one obtains blood, allows

the blood to clot, and separates the serum from the cellular components by centrifugation. In this manner, Irving's original sample was blood.

Claim Rejections - 35 USC § 103

The following is a quotation of 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.

Claims 63-68 are rejected under 35 U.S.C. 103(a) as being unpatentable over Li et al (Nature 335:414-417 (1988), cited on the IDS of 03/24/2012).

The teachings of Li have been discussed. With regard to claim 63, Li did not actually distribute into 500 assay samples. With regard to claims 64, 67 and 68, Li did not distribute into 1000 assay samples.

However, Li expressly suggested analyzing 500 assay samples; page 416, last paragraph: "With PCR, we can envisage typing as many as 500 meiotic products in a week."

It would have been *prima facie* obvious to one of ordinary skill in the art at the time the invention was made to distribute 500, or even 1000 individual sperm and assay according to Li's technique. One would have been motivated to do so because Li stated (page 416, first paragraph of "Discussion"): "A significant advantage of the approach described here is that a large number of meiotic products can be examined from a single individual allowing determination of the recombination frequency of recombination between genetic markers which are

physically very close." Li's express contemplate of 500 individual meiotic event certainly renders claim 63 obvious, and, by simple extrapolation, the subject matter of claims 64, 65, 67 and 68, which merely require more assay samples (i.e. 1000).

Claims 50, 51 and 55-68 are rejected under 35 U.S.C. 103(a) as being unpatentable over Irving et al (The Journal of Infectious Diseases 180:27-34, July 1999) in view of Simmonds et al (Journal of Virology 64(2):864-872 (1990)).

The teachings of Irving have been discussed.

With regard to claims 55-65, it would have been obvious to one of skill in the art at the time the invention was made to make up to 1000 (or more) assay samples in order to obtain more virus templates for sequencing, in order to discover more viral variants within the sample.

With regard to claims 50 and 66, Irving did not specifically say that the "end-point dilution" (page 28, column 1, last [full] paragraph) used to obtain single molecules for DNA sequencing was a dilution that produced, on average, 0.5 templates per assay sample. However, Irving cites to Simmonds (ref. 12). Simmonds states (page 871, last paragraph):

cule is present will usually be greater than this. From the Poisson distribution, when 50% of reactions are positive, about one-third of the positive reactions will contain two template molecules; when 25% are positive, about one-eighth of the positive reactions will contain two templates.

From this it is clear that the number of assay samples containing a single molecule would be dependent upon how much dilution of the original sample was made (and, consequently, on the average number of template molecules per assay sample). As such, the average number of template molecules per assay samples is a variable affecting the number of assay samples containing a single template, which was the desire of Irving. Therefore, the average number of template molecules per assay sample was a variable subject to routine optimization (MPEP 2144.05(II)(B)). "Where the general conditions in a claim are disclosed in the prior art, it is not inventive to discover the optimum or workable ranges by routine experimentation." *In re Aller*, 220 F.2d 454, 456, 105 USPQ 233, 235 (CCPA 1955).

With regard to claims 51 and 67, in arriving at an average of 0.5 molecules per assay sample, one would also have arrived at a state wherein between 0.1 and 0.9 assay samples produced an amplification product, as 50% is between 10% and 90%.

Conclusion

No claims are free of the prior art.

Any inquiry concerning this communication or earlier communications from the examiner should be directed to SAMUEL WOOLWINE whose telephone number is (571)272-1144. The examiner can normally be reached on Mon-Fri 9:00am-5:00pm.

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.

/Samuel Woolwine/
Primary Examiner

Notice of References Cited	Application/Control No. 13/071,105	Applicant(s)/Patent Under Reexamination VOGELSTEIN ET AL.	
	Examiner SAMUEL WOOLWINE	Art Unit 1637	Page 1 of 1

U.S. PATENT DOCUMENTS

*		Document Number Country Code-Number-Kind Code	Date MM-YYYY	Name	Classification
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NON-PATENT DOCUMENTS

*		Include as applicable: Author, Title Date, Publisher, Edition or Volume, Pertinent Pages)
	U	Irving et al. TT Virus Infection in Patients with Hepatitis C: Frequency, Persistence, and Sequence Heterogeneity. The Journal of Infectious Diseases 180:27-34, July 1999.
	V	Simmonds et al. Human immunodeficiency virus-infected individuals contain provirus in small numbers of peripheral mononuclear cells and at low copy numbers. Journal of Virology 64(2):864-872 (1990).
	W	
	X	

*A copy of this reference is not being furnished with this Office action. (See MPEP § 707.05(a).)
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	13071105	VOGELSTEIN ET AL.
	Examiner	Art Unit
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SEARCH NOTES		
Search Notes	Date	Examiner
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INFORMATION DISCLOSURE STATEMENT BY APPLICANT (**Not for submission under 37 CFR 1.99)**		
	Application Number	
	Filing Date	2011-03-16
	First Named Inventor	Bert Vogelstein et al.
	Art Unit	
	Examiner Name	
	Attorney Docket Number	001107.00866

U.S.PATENTS						Remove
Examiner Initial*	Cite No	Patent Number	Kind Code[1]	Issue Date	Name of Patentee or Applicant of cited Document	Pages,Columns,Lines where Relevant Passages or Relevant Figures Appear
	1	5213961	A	1993-05-15	Bunn et al.	
	2	5736333	A	1998-04-07	Livak et al.	
	3	5518901	A	1996-05-21	Murtagh	
	4	5804383	A	1998-09-08	Gruenert et al.	
	5	5858663	A	1999-01-12	Nisson et al.	
	6	5670325	A	1997-09-23	Lapidus et al.	
	7	6037130	A	2000-03-14	Tyagi et al.	
	8	5925517	A	1999-07-20	Tyagi et al.	

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INFORMATION DISCLOSURE STATEMENT BY APPLICANT (Not for submission under 37 CFR 1.99)		
	Application Number	
	Filing Date	2011-03-16
	First Named Inventor	Bert Vogelstein et al.
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	Examiner Name	
	Attorney Docket Number	001107.00866

	9	5928870	A	1999-07-27	Lapidus et al.	
	10	6020137	A	2000-02-01	Lapidus et al.	
	11	6143496	A	2000-11-07	Brown et al.	
	12	6291163	B1	2001-09-18	Sidransky	

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	1	95/13399	WO	A1	1995-05-18			☐
	2	99/13113	WO	A1	1999-03-18			☐

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	Application Number	
	Filing Date	2011-03-16
	First Named Inventor	Bert Vogelstein et al.
	Art Unit	
	Examiner Name	
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	3	0643140	EP	A1	1995-03-15	Canon Kabushiki Kaisha		☐

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Examiner Initials*	Cite No	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, pages(s), volume-issue number(s), publisher, city and/or country where published.	T5
	1	LOUGHLIN ET AL., "Association of the Interleukin-1 Gene Cluster on Chromosome 2q13 With Knee Osteoarthritis," Arthritis & Rheumatism, June 2002, 46(6):1519-1527	☐
	2	P. J. SYKES, "Quantitation of Targets for PCR by Use of Limiting Dilution," BioTechniques, 1992, Vol. 13, No. 3, pp. 444-449	☐
	3	A. PIATEK ET AL., "Molecular Beacon Sequence Analysis for Detecting Drug Resistance in Mycobacterium Tuberculosis," Nature Biotechnology, April 1998, Vol. 16, No. 4. pp. 359-363	☐
	4	S. TYAGI ET AL., "Multicolor Molecular Beacons for allele discrimination," Nature Biotechnology, January 1998, Vol. 16, No. 1, pp. 303-308	☐
	5	J. A.M. VET ET AL., "Multilex Detection of Four Pathogenic Retroviruses Using Molecular Beacons," Proceedings of the National Academy of Sciences of the United States", May 25, 1999, Vol. 96, No. 11, pp. 6394-6399	☐
	6	S. TYAGI ET AL., "Molecular Beacons: probes that Fluoresce Upon Hybridization," Nature Biotechnology, 1996, Vol. 14, No. 3, pp. 303-308	☐
	7	W. P. HALFORD ET AL., "The Inherent Quantitative Capacity of the Reverse Transcription-Polymerase Chain Reaction," Analytical Biochemistry, January 15, 1999, Vol. 266, No. 2, pp. 181-191	☐
	8	B. VOGELSTEIN ET AL., "Digital PCR," Proceedings of the National Academy of Sciences of the United States, August 3, 1999, Vol. 96, No. 16, pp. 9236-9241	☐

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	Filing Date	2011-03-16
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	9	K. D.E. EVERETT ET AL., "Identification of Nine Species of the Chlamydiaceae Using PCR-RFLP," Int. J. Syst. Bacteriol., April 1999, Vol. 49, No. 2, pp. 803-813	☐
	10	D. G. MONCKTON ET AL., "Minisatellite "Isoallele" Discrimination in Pseudohomozygotes by Single Molecule PCR and Variant Repeat Mapping," Genomics, 1991, Vol. 11, pp. 465-467	☐
	11	G. RUANO ET AL., "Haplotype of Multiple Polymorphisms Resolved by Enzymatic Amplification of Single DNA Molecules," Proc. National Science USA, 1990, pp. 6296-6300	☐
	12	W. NAVIDI ET AL., "Using PCR in Preimplantation Genetic Disease Diagnosis," Human Reproduction, 1991, Vol. 6, No. 6, pp. 836-849	☐
	13	H. LI ET AL., "Amplification and Analysis of DNA Sequences in Single Human Sperm and Diploid Cells," Nature, September 29, 1988, Vol. 335, pp. 414-417	☐
	14	L. ZHANG ET AL., "Whole Genome Amplification from a Single Cell: Implications for Genetic Analysism," Proc. National Science USA, July 1992, Vol. 89, pp. 5847-5851	☐
	15	D. SIDRANSKY ET AL., "Clonal Expansion of p53 Mutant Cells is Associated with Brain Tumour Progression," Nature, February 27, 1992, pp. 846-847	☐
	16	A. J. JEFFREYS ET AL., "Mutation Processes at Human Minisatellites," Electophoresis, 1995, pp. 1577-1585	☐
	17	C. SCHMITT ET AL., "High Sensitive DNA Typing Approaches for the Analysis of Forensic Evidence: Comparison of Nested Variable Number of Tandem Repeats (VNTR) Amplification and a Short Tandem Repeats (STR) Polymorphism," Forensic Science International, 1994, Vol. 66, pp. 129-141	☐
	18	P. M. LIZARDI ET AL., "Mutation Detection and Single-Molecule Counting Using Isothermal Rolling-Circle Amplification," Nature Genetics, July 1998, Vol. 19, pp. 225-232	☐
	19	R. PARSONS ET AL., "Mismatch Repair Deficiency in Phenotypically Normal Human Cells," Science, May 5, 1995, Vol. 268, pp. 738-740	☐

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	20	MARRAS ET AL., "Multiplex Detection of Single-Nucleotide Variations Using Molecular Beacons," Genetic Analysis: Biomolecular Engineering, Feb. 1999, Vo. 14, pp. 151-156	☐
	21	WHITCOMB ET AL., "Detection of PCR Products Using Self-Probing Amplicons and Fluorescence," Nature Biotechnology, August 1999, Vol. 17, pp. 804-807	☐
	22	M.J. BRISCO ET AL., "Detection and Quantitation of Neoplastic Cells in Acute Lymphoblastic Leukemia, by Use of the Polymerase Chain Reaction," British Journal of Haematology, 1991, Vol. 79, pp. 211-217	☐
	23	M. J. BRISCO ET AL., "Outcome Prediction in Childhood Acute Lymphoblastic Leukemia by Molecular Quantification of Residual Disease at the End of Induction," The Lancet, January 22, 1994, Vol. 343, pp. 196-200	☐
	24	Notice of Reasons for Rejection dispatched April 28, 2010 in Japanese Application No. 2001-513641 and English translation thereof.	☐
	25	Stephens, J. Clairborne, et al. "Theoretical underpinning of the Single-Molecular-Dilution (SMD) Method of Direct Haplotype Resolution," Am. J. Hum. Gen., Vol. 46, pp. 1149-1155 (1990).	☐
	26	NEWTON, PCR Essential Data, pages 51-52, 1995	☐
	27	Office Action dated June 11, 2010, in co-pending application 11/709,742	☐
	28	Office Action dated December 29, 2009 in co-pending application 11/709,742	☐
	29	Office Action dated September 18, 2009 in co-pending application 11/709,742	☐
	30	Office Action dated June 5, 2009 in co-pending application 11/709,742	☐

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EAST Search History

EAST Search History (Prior Art)

Ref #	Hits	Search Query	DBs	Default Operator	Plurals	Time Stamp
L1	2449	(sample with (split splitting divide divided dividing dilute diluting diluted dilution)) same ((fragment molecule template) near5 (single one "less than" "more than" "greater than" "fewer than" fewer less))	US-PGPUB; USPAT; USOCR; FPRS; EPO; JPO; DERWENT; IBM_TDB	OR	ON	2012/10/02 15:01
L2	361	l1 and (@ad<"19990802" @pd<"19990802")	US-PGPUB; USPAT; USOCR; FPRS; EPO; JPO; DERWENT; IBM_TDB	OR	ON	2012/10/02 15:01
L3	12232	rare near5 (sequence target mutation variant variation polymorphism)	US-PGPUB; USPAT; USOCR; FPRS; EPO; JPO; DERWENT; IBM_TDB	OR	ON	2012/10/02 15:02
L4	14	l2 and l3	US-PGPUB; USPAT; USOCR; FPRS; EPO; JPO; DERWENT; IBM_TDB	OR	ON	2012/10/02 15:02
S2	4	("7915015" "7824889" "6753147" "6440706").PN.	USPAT	OR	OFF	2012/10/01 07:49
S3	1	("20080287318").PN.	US-PGPUB; USPAT	OR	OFF	2012/10/01 15:54
S4	1132	"limiting dilution" same pcr	US-PGPUB; USPAT; USOCR; FPRS; EPO; JPO; DERWENT; IBM_TDB	OR	OFF	2012/10/01 16:52
S5	123	S4 and (@ad<"19990802" @pd<"19990802")	US-PGPUB; USPAT; USOCR; FPRS; EPO; JPO; DERWENT; IBM_TDB	OR	OFF	2012/10/01 17:04
S6	85	S5 and sequencing	US-PGPUB; USPAT; USOCR; FPRS; EPO;	OR	OFF	2012/10/01 17:04

			JPO; DERWENT; IBM_TDB			
S7	26	S5 and (sequencing sequenced) with ((pcr amplification) near2 product)	US-PGPUB; USPAT; USOCR; FPRS; EPO; JPO; DERWENT; IBM_TDB	OR	ON	2012/10/01 17:05
S8	4	(vogelstein kinzler).in. and (dilut$3 distribut$3).clm. and (sequencing (determin$5 near2 sequence)).clm. and (samples aliquots portions tubes wells).clm.	US-PGPUB; USPAT; USOCR; FPRS; EPO; JPO; DERWENT; IBM_TDB	OR	ON	2012/10/01 17:13
S9	289	((distributing diluting aliquotting dividing splitting) with (dna nucleic sample)) same (amplif$7 pcr) same (sequencing sequenced)	US-PGPUB; USPAT; USOCR; FPRS; EPO; JPO; DERWENT; IBM_TDB	OR	ON	2012/10/01 17:31
S10	16	S9 and (@ad<"19990802" @pd<"19990802")	US-PGPUB; USPAT; USOCR; FPRS; EPO; JPO; DERWENT; IBM_TDB	OR	OFF	2012/10/01 17:31
S11	1337	((distributing diluting aliquotting dividing splitting) with (dna nucleic sample)) same (amplif$7 pcr) and (sequencing sequenced)	US-PGPUB; USPAT; USOCR; FPRS; EPO; JPO; DERWENT; IBM_TDB	OR	ON	2012/10/01 17:32
S12	312	S11 and ((less fewer) near5 molecules)	US-PGPUB; USPAT; USOCR; FPRS; EPO; JPO; DERWENT; IBM_TDB	OR	ON	2012/10/01 17:33
S13	8	S12 and (@ad<"19990802" @pd<"19990802")	US-PGPUB; USPAT; USOCR; FPRS; EPO; JPO; DERWENT; IBM_TDB	OR	OFF	2012/10/01 17:33
S18	506	(rare adj1 (sequence target mutation)) and ((pcr "polymerase chain") same (dilution diluting diluted))	US-PGPUB; USPAT; USOCR; FPRS; EPO; JPO; DERWENT; IBM_TDB	OR	ON	2012/10/01 22:55
S19	56	S18 and (@ad<"19990802" @pd<"19990802")	US-PGPUB; USPAT;	OR	ON	2012/10/01 22:55

			USOCR; FPRS; EPO; JPO; DERWENT; IBM_TDB			
S20	12	("5213961" | "5518901" | "5670325" | "5736333" | "5804383" | "5858663" | "5925517" | "5928870" | "6020137" | "6037130" | "6143496" | "6291163").PN.	US-PGPUB; USPAT	OR	OFF	2012/10/01 23:07
S21	10	S20 and (dilution diluted diluting)	US-PGPUB; USPAT	OR	OFF	2012/10/01 23:08
S22	234225	pcr and (sequencing sequenced sequence)	US-PGPUB; USPAT; USOCR; FPRS; EPO; JPO; DERWENT; IBM_TDB	OR	ON	2012/10/02 12:06
S23	49987	S22 and (sample with (split splitting divide divided dividing dilute diluting diluted dilution))	US-PGPUB; USPAT; USOCR; FPRS; EPO; JPO; DERWENT; IBM_TDB	OR	ON	2012/10/02 12:07
S24	33237	S23 and ((molecule template) near5 (single one "less than" "more than" "greater than" "fewer than" fewer less))	US-PGPUB; USPAT; USOCR; FPRS; EPO; JPO; DERWENT; IBM_TDB	OR	ON	2012/10/02 12:09
S25	17275	S23 and (((fragment molecule template) near5 (single one "less than" "more than" "greater than" "fewer than" fewer less)) same (pcr amplif$7))	US-PGPUB; USPAT; USOCR; FPRS; EPO; JPO; DERWENT; IBM_TDB	OR	ON	2012/10/02 12:09
S26	1366	(sample with (split splitting divide divided dividing dilute diluting diluted dilution)) same (((fragment molecule template) near5 (single one "less than" "more than" "greater than" "fewer than" fewer less)) same (pcr amplif$7))	US-PGPUB; USPAT; USOCR; FPRS; EPO; JPO; DERWENT; IBM_TDB	OR	ON	2012/10/02 12:10
S27	60	S26 and (@ad<"19990802" @pd<"19990802")	US-PGPUB; USPAT; USOCR; FPRS; EPO; JPO; DERWENT; IBM_TDB	OR	ON	2012/10/02 12:11
S28	1	ruano.in. and ("single molecule" "single-molecule") adj1 dilution	US-PGPUB; USPAT; USOCR; FPRS; EPO; JPO; DERWENT; IBM_TDB	OR	OFF	2012/10/02 12:30

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EAST Search History

EAST Search History (Prior Art)

Ref #	Hits	Search Query	DBs	Default Operator	Plurals	Time Stamp
L1	104	(griffiths tawfik).in. and emulsion and pcr	US-PGPUB; USPAT; USOCR; FPRS; EPO; JPO; DERWENT; IBM_TDB	OR	ON	2012/10/02 17:49
L2	7	l1 and (@ad<"19990802")	US-PGPUB; USPAT; USOCR; FPRS; EPO; JPO; DERWENT; IBM_TDB	OR	ON	2012/10/02 17:50
L3	6	l2 and (sequenced sequencing)	US-PGPUB; USPAT; USOCR; FPRS; EPO; JPO; DERWENT; IBM_TDB	OR	ON	2012/10/02 17:50

10/2/2012 5:51:13 PM
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PATENT

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re Application of	)	
	)	
Bert VOGELSTEIN et al.	)	Examiner: WOOLWINE, Samuel C.
	)	
Serial No. 13/071,105	)	
	)	Group Art Unit: 1637
	)	
Filed: March 24, 2011	)	Confirmation No. 3361
	)	
For: **DIGITAL AMPLIFICATION**	)	Atty. Dkt. No. 001107.00866

RESPONSE TO OFFICE ACITON

Commissioner of Patents
P.O. Box 1450
Alexandria, VA 22313-1450

Sir:

In response to the office action mailed October 10, 2012, applicants request entry of the amendment and reconsideration of the patentability of the claims in view of the remarks.

A petition for a two-month extension of time to an including March 11, 2013 accompanies this submission. The Commissioner is authorized to charge any fees which may be required or credit any overpayment to our Deposit Account 19-0733.

IN THE CLAIMS

Please replace the following claim set for that currently of record.

1. -48. (Cancelled)

49. (Proposed amendment) A method for detecting quantity of a genetic sequence in a mixed population of human genomic nucleic acid sequences comprising at least a first and a second human genomic sequence, wherein the first sequence is a wild-type sequence of an allele and a second sequence is a mutant sequence of the allele, comprising:

distributing or diluting a mixed population of cell-free, human genomic nucleic acid ~~sequences~~ template molecules into a set comprising at least ten assay samples such that said at least ten assay samples each comprises less than ten template molecules;

amplifying the template molecules in the assay samples, wherein an assay sample with a single template molecule forms homogeneous amplification products in the assay sample;

analyzing by determining nucleic acid sequence of amplification products ~~from an assay sample~~ in the assay samples of the set with homogeneous amplification products to determine a first number of assay samples which contain the first sequence and a second number of assay samples which contain the second sequence;

comparing the first number to the second number to ascertain a ratio which reflects the composition of the mixed population;

identifying a mutation in the mixed population if a statistically significant fraction of samples comprises the second sequence.

50. (Currently amended) The method of claim 49 wherein ~~each of~~ the assay samples of the set have ~~has~~ on average 0.5 molecules of template.

51. (Previously Presented) The method of claim 49 wherein between 0.1 and 0.9 of the assay samples yield an amplification product.

52. (Previously Presented) The method of claim 49 wherein the mixed population of nucleic acid sequences is distributed or diluted to a single template molecule level in the assay samples.

53. (Previously Presented) The method of claim 49 wherein the mixed population of nucleic acid sequences is from a tissue or body sample.

54. (Previously Presented) The method of claim 49 wherein the mixed population of nucleic acids sequences is from a sample selected from the group consisting of stool, blood, and lymph nodes.

55. (Previously Presented) The method of claim 49 wherein the mixed population of nucleic acids sequences is distributed or diluted such that at least fifteen assay samples comprise less than ten template molecules.

56. (Previously Presented) The method of claim 49 wherein the mixed population of nucleic acids sequences is distributed or diluted such that at least twenty assay samples comprise less than ten template molecules.

57. (Previously Presented) The method of claim 49 wherein the mixed population of nucleic acids sequences is distributed or diluted such that at least twenty-five assay samples comprise less than ten template molecules.

58. (Previously Presented) The method of claim 49 wherein the mixed population of nucleic acids sequences is distributed or diluted such that at least thirty assay samples comprise less than ten template molecules.

59. (Previously Presented) The method of claim 49 wherein the mixed population of nucleic acids sequences is distributed or diluted such that at least forty assay samples comprise less than ten template molecules.

60. (Previously Presented) The method of claim 49 wherein the mixed population of nucleic acids sequences is distributed or diluted such that at least fifty assay samples comprise less than ten template molecules.

61. (Previously Presented) The method of claim 49 wherein the mixed population of nucleic acids sequences is distributed or diluted such that at least seventy-five assay samples comprise less than ten template molecules.

62. (Previously Presented) The method of claim 49 wherein the mixed population of nucleic acids sequences is distributed or diluted such that at least one hundred assay samples comprise less than ten template molecules.

63. (Previously Presented) The method of claim 49 wherein the mixed population of nucleic acids sequences is distributed or diluted such that at least five hundred assay samples comprise less than ten template molecules.

64. (Previously Presented) The method of claim 49 wherein the mixed population of nucleic acids sequences is distributed or diluted such that at least one thousand assay samples comprise less than ten template molecules.

65. (Previously Presented) The method of claim 49 wherein the mixed population of nucleic acids sequences is distributed or diluted such that at least one thousand assay samples are diluted to a single template molecule level.

66. (Previously Presented) The method of claim 49 wherein the mixed population of nucleic acids sequences is distributed or diluted such that at least one thousand assay samples has on average 0.5 molecules of template.

67. (Previously Presented) The method of claim 49 wherein the mixed population of nucleic acids sequences is distributed or diluted such that between 0.1 and 0.9 of at least one thousand assay samples yield an amplification product.

68. (Previously Presented) The method of claim 49 wherein the mixed population of nucleic acids sequences is distributed or diluted such that one half of at least one thousand assay samples have one template molecule.

Remarks

The amendments to claim 49 are fully supported and do not add new matter. Quantitative analysis is taught at page 9, last paragraph. First and second sequences as mutant and wild-type sequence of an allele are taught at page 6, last paragraph. Sequencing and determining ratios to determine a mutation is taught at the paragraph spanning pages 7 and 8. The step of identifying a mutation is also taught at the paragraph spanning pages 7 and 8. Dilution/distribution of cell-free nucleic acids is taught *inter alia* at page 11, first full paragraph. The amendment to claim 49 to recite human genomic sequences is supported at page 17, lines 1-3.

The rejection under § 112, second paragraph

Claim 50 has been amended to address the unclear claim language. Please withdraw the rejection under § 112, second paragraph in view of the amendment.

Rejection under § 102(b)

Claims 49, 51-53, and 55-62 stand rejected as anticipated by Li. Li is cited as teaching dilution of a sample comprising sperm and subsequently lysing the sperm and amplifying. This is distinct from dilution of a cell-free sample of nucleic acids. Li does not teach dilution of a cell-free sample of nucleic acids. Thus Li does not anticipate the claimed invention. Please withdraw the rejection under § 102 in view of the amendment to recite dilution of a cell-free nucleic acid population.

Rejection under § 102(a)

Claims 49, and 52-54 stand rejected as anticipated by Irving. Irving is cited as teaching amplification of end-point dilution aliquots and sequencing the amplification products. Irving studies the variants in a population of TTV virus in a single individual. Irving does not identify human genomic mutant and wild-type sequences, nor does Irving use the fraction of mutant sequences to identify mutations. For at least these reasons, Irving does not anticipate claims 49 and 52-54.

The first rejection under § 103(a)

Claims 63-68 are rejected as obvious over Li. Claims 63-68 specify the number of assay samples into which the nucleic acids are distributed or diluted. Li is cited as suggesting typing as many as 500 products in a week. However, Li, did not suggest the dilution or distribution of cell-free DNA. Li's technique relied on dilution of whole, intact sperm cells. For at least this reason, Li does not render obvious the subject matter of claims 63-68.

The second rejection under § 103(a)

Claims 50, 51, and 55-68 stand rejected as obvious over Irving in view of Simmonds. As discussed above, Irving taught detection of variants within a virus population from a single infected patient.

With regard to claims 55-65 which recite various numbers of assay samples between 15 and 1000, the Patent and Trademark Office urges that although Irving did not actually report such large experiments, it would have been obvious to do them in order to find more variants. It is not clear why one of ordinary skill in the art would want to find more variants. The rejection does not identify what unanswered question in Irving's study more variants would address. As it stand, the rejection lacks any articulated reason why one of skill would do the proposed experiments.

With regard to claims 50 and 66 which recite a particular level of dilution/distribution, the Patent and Trademark Office urges that Simmonds teaches that the number of templates in an assay is a function of the level of dilution. Neither Simmonds nor Irving teaches that the recited average of 0.5 molecules is a desired level. Simmonds teaches 0.33 and 0.125, but not 0.5. Even if one accepts for the sake of argument that Irving did want 1 template per assay, the combination of Simmonds with Irving does not teach an average of 0.5. The Patent and Trademark Office bootstraps its argument by suggesting that 0.5 molecules is mere optimization. But neither Simmonds nor Irving is alleged to teach for what the parameter is being optimized.

The Patent and Trademark Office's rejection with regard to claims 51 and 67 is derivative of the rejection of claims 55-65. But that rejection is deficient, as noted.

Perhaps more significantly, Irving does not identify mutant and wild-type sequences, nor does Irving use the fraction of mutant sequences to identify mutations which occurred *in vivo* rather than *in silico*. All of claims 50, 51, and 55-68 depend from claim 49 which identifies the the fraction of allegedly mutant sequence which is used to determine whether the mutation is "real" or artifactual, *i.e.,* generated *in vivo* or generated in the amplification reaction.

Neither Irving nor Simmonds suggests such steps. For that reason as well, the method of claims 50, 51, and 55-68 are not obvious over Irving in view of Simmonds.

Please withdraw the rejection under § 103, in view *inter alia* of the amendments to claim 49.

Respectfully submitted,

Date: *March 11, 2013*

By: */Sarah A. Kagan/*
Sarah A. Kagan
Registration No. 32,141

Banner & Witcoff, Ltd.
Customer No. 11332

PTO/SB/22 (09-11)
Approved for use through 07/31/2012. OMB 0651-0031
U.S. Patent and Trademark Office; U.S. DEPARMENT OF COMMERCE

PETITION FOR EXTENSION OF TIME UNDER 37 CFR 1.136(a)	Docket Number (Optional) 001107.00866
Application Number 13/071,105	Filed March 24, 2011
For Digital Amplification	
Art Unit 1637	Examiner Samuel C. Woolwine

This is a request under the provisions of 37 CFR 1.136(a) to extend the period for filing a reply in the above identified application.

The requested extension and fee are as follows (check time period desired and enter the appropriate fee below):

		Fee	Small Entity Fee	
☐	One month (37 CFR 1.17(a)(1))	$150	$75	$
☑	Two months (37 CFR 1.17(a)(2))	$560	$280	$ 570.00
☐	Three months (37 CFR 1.17(a)(3))	$1270	$635	$
☐	Four months (37 CFR 1.17(a)(4))	$1980	$990	$
☐	Five months (37 CFR 1.17(a)(5))	$2690	$1345	$

☐ Applicant claims small entity status. See 37 CFR 1.27.

☐ A check in the amount of the fee is enclosed.

☐ Payment by credit card. Form PTO-2038 is attached.

☐ The Director has already been authorized to charge fees in this application to a Deposit Account.

☑ The Director is hereby authorized to charge any fees which may be required, or credit any overpayment, to Deposit Account Number 190733.

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.

I am the

☐ applicant/inventor.

☐ assignee of record of the entire interest. See 37 CFR 3.71.
Statement under 37 CFR 3.73(b) is enclosed (Form PTO/SB/96).

☑ attorney or agent of record. Registration Number 32,141

☐ attorney or agent under 37 CFR 1.34.
Registration number if acting under 37 CFR 1.34 _____

/Sarah A. Kagan/ — Signature

11 March 2013 — Date

Sarah A. Kagan — Typed or printed name

(202) 824-3000 — Telephone Number

NOTE: Signatures of all the inventors or assignees of record of the entire interest or their representative(s) are required. Submit multiple forms if more than one signature is required, see below.

☑ Total of 1 forms are submitted.

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.

Electronic Patent Application Fee Transmittal

Application Number:	13071105
Filing Date:	24-Mar-2011
Title of Invention:	Digital Amplification
First Named Inventor/Applicant Name:	Bert VOGELSTEIN
Filer:	Sarah Anne Kagan./Jennifer Hazzard
Attorney Docket Number:	001107.00866

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:				
Extension - 2 months with $0 paid	1252	1	570	570

Description	Fee Code	Quantity	Amount	Sub-Total in USD($)
Miscellaneous:				
			Total in USD ($)	**570**

Electronic Acknowledgement Receipt

EFS ID:	15168667
Application Number:	13071105
International Application Number:	
Confirmation Number:	3361
Title of Invention:	Digital Amplification
First Named Inventor/Applicant Name:	Bert VOGELSTEIN
Customer Number:	11332
Filer:	Sarah Anne Kagan./Jennifer Hazzard
Filer Authorized By:	Sarah Anne Kagan.
Attorney Docket Number:	001107.00866
Receipt Date:	11-MAR-2013
Filing Date:	24-MAR-2011
Time Stamp:	12:43:15
Application Type:	Utility under 35 USC 111(a)

Payment information:

Submitted with Payment	yes
Payment Type	Deposit Account
Payment was successfully received in RAM	$570
RAM confirmation Number	22590
Deposit Account	190733
Authorized User	

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

Charge any Additional Fees required under 37 C.F.R. Section 1.16 (National application filing, search, and examination fees)

Charge any Additional Fees required under 37 C.F.R. Section 1.17 (Patent application and reexamination processing fees)

Charge any Additional Fees required under 37 C.F.R. Section 1.19 (Document supply fees) Charge any Additional Fees required under 37 C.F.R. Section 1.20 (Post Issuance fees) Charge any Additional Fees required under 37 C.F.R. Section 1.21 (Miscellaneous fees and charges)

File Listing:

Document Number	Document Description	File Name	File Size(Bytes)/ Message Digest	Multi Part /.zip	Pages (if appl.)
1		Response-to-NFOA-as-filed.PDF	95962 c2b10b9891e55742fa615f10b56cd60a6bc4bf0a	yes	8

	Multipart Description/PDF files in .zip description		
	Document Description	**Start**	**End**
	Amendment/Req. Reconsideration-After Non-Final Reject	1	1
	Claims	2	5
	Applicant Arguments/Remarks Made in an Amendment	6	8

Warnings:					
Information:					
2	Extension of Time	Petition-for-EOT.PDF	289378 8919f47c7ad4711247bcaa1cf63e1efecb836292	no	2
Warnings:					
Information:					
3	Fee Worksheet (SB06)	fee-info.pdf	30272 92ce82a7a1eb8b0196183f2ecec64f34b3c693e2	no	2
Warnings:					
Information:					
		Total Files Size (in bytes):	415612		

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 (07-06)
Approved for use through 1/31/2007. OMB 0651-0032
U.S. Patent and Trademark Office; U.S. DEPARTMENT OF COMMERCE

PATENT APPLICATION FEE DETERMINATION RECORD

Substitute for Form PTO-875

Application or Docket Number	Filing Date	☐ To be Mailed
13/071,105	03/24/2011	

APPLICATION AS FILED – PART I

FOR	(Column 1) NUMBER FILED	(Column 2) NUMBER EXTRA	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	
☐ SEARCH FEE (37 CFR 1.16(k), (i), or (m))	N/A	N/A	N/A			N/A	
☐ EXAMINATION FEE (37 CFR 1.16(o), (p), or (q))	N/A	N/A	N/A			N/A	
TOTAL CLAIMS (37 CFR 1.16(i))	minus 20 =	*	X $ =		OR	X $ =	
INDEPENDENT CLAIMS (37 CFR 1.16(h))	minus 3 =	*	X $ =			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 $250 ($125 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			TOTAL	

APPLICATION AS AMENDED – PART II

AMENDMENT 03/11/2013	(Column 1) CLAIMS REMAINING AFTER AMENDMENT		(Column 2) HIGHEST NUMBER PREVIOUSLY PAID FOR	(Column 3) PRESENT EXTRA	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))	* 20	Minus	** 68	= 0	X $ =		OR	X $62=	0
Independent (37 CFR 1.16(h))	* 1	Minus	***6	= 0	X $ =		OR	X $250=	0
☐ 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	0

AMENDMENT	(Column 1) CLAIMS REMAINING AFTER AMENDMENT		(Column 2) HIGHEST NUMBER PREVIOUSLY PAID FOR	(Column 3) PRESENT EXTRA	RATE ($)	ADDITIONAL FEE ($)		RATE ($)	ADDITIONAL FEE ($)
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 number found in the appropriate box in column 1.

Legal Instrument Examiner:
/SHARON HARRIS/

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.
13/071,105	03/24/2011	Bert VOGELSTEIN	001107.00866	3361

11332 7590 06/27/2013

Banner & Witcoff, Ltd.
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EXAMINER	
WOOLWINE, SAMUEL C	
ART UNIT	PAPER NUMBER
1637	

MAIL DATE	DELIVERY MODE
06/27/2013	PAPER

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.

Office Action Summary	Application No. 13/071,105	Applicant(s) VOGELSTEIN ET AL.	
	Examiner SAMUEL WOOLWINE	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* MONTH(S) OR THIRTY (30) DAYS, WHICHEVER IS LONGER, 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 *11 March 2013*.

☐ 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) *49-68* 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) *49-68* 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.

Interim copies:

a)☐ All b)☐ Some c)☐ None of the: Interim copies of the priority documents have been received.

Attachment(s)

1) ☒ Notice of References Cited (PTO-892)

2) ☐ Information Disclosure Statement(s) (PTO/SB/08) Paper No(s)/Mail Date _____.

3) ☐ Interview Summary (PTO-413) Paper No(s)/Mail Date. _____ .

4) ☐ Other: _____.

DETAILED ACTION

Status

Applicant's reply filed 03/11/2013 is acknowledged. Claims 49-68 are pending. With regard to the Office action mailed 10/12/2012:

The rejection of claim 50 under 35 USC 112, 2nd paragraph, is withdrawn in view of the amendment.

The rejections under 35 USC 102 and 103 over Li are withdrawn in view of the amendment introducing the limitation "cell-free".

The rejections under 35 USC 102 and 103 over Irving are withdrawn in view of the amendment introducing the limitation "human".

New rejections are set forth below in response to the amendment.

Claim Rejections - 35 USC § 103

The following is a quotation of 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.

The factual inquiries set forth in *Graham* v. *John Deere Co.*, 383 U.S. 1, 148 USPQ 459 (1966), that are applied for establishing a background for determining obviousness under 35 U.S.C. 103(a) are summarized as follows:

1. Determining the scope and contents of the prior art.
2. Ascertaining the differences between the prior art and the claims at issue.
3. Resolving the level of ordinary skill in the pertinent art.
4. Considering objective evidence present in the application indicating obviousness or nonobviousness.

This application currently names joint inventors. In considering patentability of the claims under 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 35 U.S.C. 103(c) and potential 35 U.S.C. 102(e), (f) or (g) prior art under 35 U.S.C. 103(a).

Claims 49 and 51-53 are rejected under 35 U.S.C. 103(a) as being unpatentable over Ruano et al (PNAS 87:6296-6300 (1990), cited on the IDS of 10/10/2012).

With regard to claim 49, Ruano taught a method comprising:

distributing or diluting a mixed population of cell-free, human genomic nucleic acid template molecules into a set comprising at least ten assay samples such that said at least 10 assay samples each comprises less than ten template molecules

See figure 3a; Ruano diluted and distributed genomic DNA into ten samples (lanes 4-13) having 1 template copy.

amplifying the template molecules in the assay samples, wherein an assay sample with a single template molecule forms homogeneous amplification products in the assay sample

See figure 3a; “booster PCR. In addition, amplification of a single template molecule would inherently form a homogeneous product. Note in the caption of figure 3, Ruano states: "...while product amplified from 1000 template copies contains both

alleles (lane 1), SMD products from this series contain one or the other." SMD stands for "single molecule dilution".

analyzing by determining nucleic acid sequence of amplification products in the set with homogeneous amplification products to determine a first number of assay samples which contain the first sequence and a second number of assay samples which contain the second sequence

Ruano was amplifying a 770 bp fragment in the intergenic region between ψβ and δ, which fragment contained a polymorphic site (figure 1). One allele constitutes a TaqI restriction site, while the other does not (*id.*). Thus, the sequence of the SMD amplification product could be either of these two sequences, and Ruano determined the correct sequence of the SMD amplification product in each of the sample containing homogeneous products (i.e. lanes 5, 6, 7 and 10 of figure 3a) by analyzing TaqI digests of the amplification products (figure 3b). Ruano thereby determined that 2 of the 4 samples yielding homogeneous amplification products comprised the TaqI-positive allele, while the other 2 comprised the TaqI-negative allele.

With regard to claim 51, in Ruano's experiment (figure 3), 0.4 of the assay samples yielded an amplification product.

With regard to claim 52, Ruano diluted and distributed the mixed population down to a single template molecule level in the assay samples; see figure 3.

With regard to claim 53, while Ruano does not specifically state the type of sample, but merely that it was "genomic DNA from each individual" (page 6297, column

1, 4th paragraph entitled "Standard PCR"). However, genomic DNA from an individual would necessarily come from the individual's body.

With regard to claim 49, Ruano did not expressly teach:

comparing the first number to the second number to ascertain a ratio which reflects the composition of the mixed population;

identifying a mutation in the mixed population if a statistically significant fraction of samples comprises the second sequence.

With regard to claim 49, it would have been *prima facie* obvious to one of ordinary skill in the art at the time the invention was made to compare the number of assay samples yielding the first sequence to the number of samples yielding the second sequence to arrive at a ratio, and to identify the presence of a mutation (note that either the TaqI-positive or TaqI-negative allele can be considered "wild-type" while the other can be considered the "mutation") is a statistically significant fraction of samples comprised the second sequence. This would have been self-evident by merely looking at the results in Ruano's figure 3b. It was clearly evident that 50% (a statistically significant fraction) of the samples yielding an amplification product had the second sequence. Thus, it would have been obvious for one of ordinary skill in the art to look at the results and make a mental comparison of the number of samples yielding each allele and think: "Hmmm. Fifty percent of the samples are $TaqI^+$ and fifty percent are $TaqI^-$, so both alleles [and hence the "mutation"] are present." This simple assessment arrives at the limitations:

comparing the first number to the second number to ascertain a ratio which reflects the composition of the mixed population;

identifying a mutation in the mixed population if a statistically significant fraction of samples comprises the second sequence.

Claims 50 and 55-68 are rejected under 35 U.S.C. 103(a) as being unpatentable over Ruano et al (PNAS 87:6296-6300 (1990), cited on the IDS of 10/10/2012) as applied to claims 49 and 51-53 above, and further in view of Stephens et al (Am J Hum Gen 46:1149-1155 (1990), cited on the IDS of 10/10/2012).

With regard to claim 50, Ruano's dilution was designed to give, on average, one molecule of template per sample; see page 6297, column 2, line 6+. Ruano did not teach a desire to achieve an average of 0.5 molecules of template per sample.

With regard to claims 55-68, Ruano did not teach more than 10 samples, and so did not teach the number of samples recited in these claims.

Stephens taught theoretical considerations for single molecule dilution of nucleic acid for the purposes of trying to amplify single template molecules. In Table 1 (page 1150), Stephens estimates the probability of success (defined as having one or more of *v* vials having one but not both haplotypes present; see sentence spanning columns 1-2, page 1150). According to this table, for a given dilution (i.e. DNA concentration in haploid equivalents), the probability of success increases with an increasing number of samples (i.e. vials, *v*). Also according to this table, while a DNA concentration of 1 is optimal, a DNA concentration of 0.5 still has a very high probability of success. Thus,

Stephens establishes that both the number of samples used and the dilution (i.e. the average number of template molecules per sample) are results-effective variables. Note that Stephens' table is based on theoretical calculations, so while his figures would have provided a reasonable place to start, some actual empirical optimization would have been warranted. For this reason, claims 50 and 55-68 are *prima facie* obvious on the basis that it is *prima facie* obvious to optimize conditions known in the prior art that were results-effective variables (in this case, the number of samples and the number of template molecules per sample; see MPEP 2144.05(II)).

Claims 55-68 are rejected under 35 U.S.C. 103(a) as being unpatentable over Ruano et al (PNAS 87:6296-6300 (1990), cited on the IDS of 10/10/2012) as applied to claims 49 and 51-53 above, and further in view of Kruglyak (Nature Genetics 22:139-144, June 1999).

With regard to claims 55-68, Ruano did not teach more than 10 samples, and so did not teach the number of samples recited in these claims.

Kruglyak taught an interest in a whole-genome linkage disequilibrium map, estimating it would require analysis of about 500,000 SNPs (single nucleotide polymorphisms); see abstract. Kruglyak also taught that with lower-frequency variants (i.e. SNPs present in the population at low frequency), would require the use of haplotypes (page 141, column 1, middle paragraph). Taken together, these teachings suggest performing haplotype analysis at many different markers.

Thus, it would have been *prima facie* obvious to one of ordinary skill in the art at the time the invention was made to use the method of Ruano for haplotype analysis as many different sites throughout the genome, thus necessitating the use of up to 1000 or even more "single molecule" samples.

Claim 54 is rejected under 35 U.S.C. 103(a) as being unpatentable over Ruano et al (PNAS 87:6296-6300 (1990), cited on the IDS of 10/10/2012) as applied to claims 49 and 51-53 above, and further in view of Kulozik et al (Am J Hum Gen 39:239-244 (1986)).

The teachings of Ruano have been discussed. Ruano did not specify what type of sample the genomic DNA came from (e.g. blood, tissue, etc).

Kulozik performed haplotype analysis of the β-globin locus (see title), and used blood samples to do so (page 240, first statement under “Patients and Methods”).

It would have been *prima facie* obvious to one of ordinary skill in the art at the time the invention was made to use blood as the sample from which the DNA was obtained when practicing the method of Ruano, since blood was a common sample used in haplotyping studies (as well as numerous other types of studies).

Response to Arguments

Applicant’s arguments with respect to claims 49-68 have been considered but are moot because the arguments do not apply to any of the references being used in the current rejection.

Conclusion

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 SAMUEL WOOLWINE whose telephone number is (571)272-1144. The examiner can normally be reached on Mon-Fri 9:00am-5:00pm.

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.

/Samuel Woolwine/
Primary Examiner

Notice of References Cited	Application/Control No. 13/071,105	Applicant(s)/Patent Under Reexamination VOGELSTEIN ET AL.	
	Examiner SAMUEL WOOLWINE	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-			
	B	US-			
	C	US-			
	D	US-			
	E	US-			
	F	US-			
	G	US-			
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	I	US-			
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	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	Kruglyak, L. Prospects for whole-genome linkage disequilibrium mapping of common disease genes. Nature Genetics 22:139-144, June 1999.
	V	Kulozik et al. Geographical survey of βs-globin gene haplotypes: evidence for an independent Asian origin of the sickle-cell mutation. Am J Hum Gen 39:239-244 (1986).
	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.

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=> s (dilute or diluted or dilution or diluting or dilutes)(s)(single(3w)molecule)
L1          271 (DILUTE OR DILUTED OR DILUTION OR DILUTING OR DILUTES)(S)(SINGLE
                (3W) MOLECULE)

=> s l1 and (amplification or pcr or "polymerase chain" or amplifying or amplify or
amplified)
L2           48 L1 AND (AMPLIFICATION OR PCR OR "POLYMERASE CHAIN" OR AMPLIFYING
                OR AMPLIFY OR AMPLIFIED)

=> s l2 and py<2000
L3           21 L2 AND PY<2000
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     FILE 'MEDLINE, CAPLUS' ENTERED AT 17:27:28 ON 14 JUN 2013
L1         58105 SEA ABB=ON  PLU=ON  (SINGLE(3W) MOLECULE)
L2          1911 SEA ABB=ON  PLU=ON  (SINGLE(3W) MOLECULE)(S)(DILUTE OR
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L3           300 SEA ABB=ON  PLU=ON  L2 AND PY<2000
L4            56 SEA ABB=ON  PLU=ON  L3 AND (HAPLOTYP? OR MUTATION? OR POLYMORPH
                 ISM? OR ALLELE? OR SNP? OR HETEROZYGOUS OR HETEROZYGOSITY)
L5            37 DUP REM L4 (19 DUPLICATES REMOVED)
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Search Notes	Application/Control No.	Applicant(s)/Patent Under Reexamination
	13071105	VOGELSTEIN ET AL.
	Examiner	Art Unit
	SAMUEL WOOLWINE	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
Inventor name/keyword search in EAST, Google Scholar.	10/04/2012	SCW
Update search in STN	06/15/2013	SCW

INTERFERENCE SEARCH			
US Class/ CPC Symbol	US Subclass / CPC Group	Date	Examiner
			SCW

PTO/SB/08a (01-10)
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INFORMATION DISCLOSURE STATEMENT BY APPLICANT

(Not for submission under 37 CFR 1.99)

Application Number	13071105
Filing Date	2011-03-24
First Named Inventor	Bert VOGELSTEIN
Art Unit	1637
Examiner Name	Samuel C. Woolwine
Attorney Docket Number	001107.00866

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	1	0643140	EP	A1	1995-03-15	Yamamoto, et al.		☐

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Examiner Initials*	Cite No	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, pages(s), volume-issue number(s), publisher, city and/or country where published.	T[5]

INFORMATION DISCLOSURE STATEMENT BY APPLICANT (Not for submission under 37 CFR 1.99)		
	Application Number	13071105
	Filing Date	2011-03-24
	First Named Inventor	Bert VOGELSTEIN
	Art Unit	1637
	Examiner Name	Samuel C. Woolwine
	Attorney Docket Number	001107.00866

	1	Examiner Requisition issued dated April 12, 2013 issued by the Canadian Intellectual Property Office in Canadian Application No. 2,756,675	☐
	2	Piatek, A. et al., "Molecular beacon sequence analysis for detecting drug resistance in Mycobacterium tuberculosis", Nature Biotechnology, 16(4), Pages 359-363, April 1, 1998.	☐

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*EXAMINER: Initial if reference considered, whether or not citation is in conformance with MPEP 609. Draw line through a citation if not in conformance and not considered. Include copy of this form with next communication to applicant.

[1] See Kind Codes of USPTO Patent Documents at www.USPTO.GOV or MPEP 901.04. [2] Enter office that issued the document, by the two-letter code (WIPO Standard ST.3). [3] For Japanese patent documents, the indication of the year of the reign of the Emperor must precede the serial number of the patent document. [4] Kind of document by the appropriate symbols as indicated on the document under WIPO Standard ST.16 if possible. [5] Applicant is to place a check mark here if English language translation is attached.

INFORMATION DISCLOSURE STATEMENT BY APPLICANT (Not for submission under 37 CFR 1.99)		
	Application Number	13071105
	Filing Date	2011-03-24
	First Named Inventor	Bert VOGELSTEIN
	Art Unit	1637
	Examiner Name	Samuel C. Woolwine
	Attorney Docket Number	001107.00866

CERTIFICATION STATEMENT

Please see 37 CFR 1.97 and 1.98 to make the appropriate selection(s):

☒ That each item of information contained in the 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 the information disclosure statement. See 37 CFR 1.97(e)(1).

OR

☐ That no item of information contained in the information disclosure statement was cited in a communication from a foreign patent office in a counterpart foreign application, and, to the knowledge of the person signing the certification after making reasonable inquiry, no item of information contained in the information disclosure statement was known to any individual designated in 37 CFR 1.56(c) more than three months prior to the filing of the information disclosure statement. See 37 CFR 1.97(e)(2).

☐ See attached certification statement.

☒ The fee set forth in 37 CFR 1.17 (p) has been submitted herewith.

☐ A certification statement is not submitted herewith.

SIGNATURE

A signature of the applicant or representative is required in accordance with CFR 1.33, 10.18. Please see CFR 1.4(d) for the form of the signature.

Signature	/Sarah A. Kagan/	Date (YYYY-MM-DD)	2013-07-12
Name/Print	Sarah A. Kagan	Registration Number	32141

This collection of information is required by 37 CFR 1.97 and 1.98. 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 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: Commissioner for Patents, P.O. Box 1450, Alexandria, VA 22313-1450.**

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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.

Espacenet

Bibliographic data: EP0643140 (A1) — 1995-03-15

Determination of nucleic acid by PCR, measurement of number of microbial cells, genes, or gene-copies by PCR, and measuring-kit employed for the same.

No documents available for this priority number.

Inventor(s): YAMAMOTO NOBUKO C O CANON KABU [JP]; OKAMOTO TADASHI C O CANON KABU [JP]; TOMIDA YOSHINORI C O CANON KAB [JP]; YANO TETSUYA C O CANON KABUSHI [JP]; MIYAZAKI TAKESHI C O CANON KAB [JP]; KAWAGUCHI MASAHIRO C O CANON K [JP] ± (YAMAMOTO, NOBUKO, C/O CANON KABUSHIKI KAISHA, ; OKAMOTO, TADASHI, C/O CANON KABUSHIKI KAISHA, ; TOMIDA, YOSHINORI C/O CANON KABUSHIKI KAISHA, ; YANO, TETSUYA, C/O CANON KABUSHIKI KAISHA, ; MIYAZAKI, TAKESHI, C/O CANON KABUSHIKI KAISHA, ; KAWAGUCHI, MASAHIRO, C/O CANON KABUSHIKI KAISHA, ; YAMAMOTO, NOBUKO, ; OKAMOTO, TADASHI, ; TOMIDA, YOSHINORI, ; YANO, TETSUYA, ; MIYAZAKI, TAKESHI, ; KAWAGUCHI, MASAHIRO)

Applicant(s): CANON KK [JP] ± (CANON KABUSHIKI KAISHA)

Classification: **- international:** ***C09B57/00; C12Q1/68; G01N33/52;*** (IPC1-7): C09B57/00; C12Q1/68; G01N33/52
- cooperative: **C09B57/00**; **C12Q1/6851**; **G01N33/52**

Application number: EP19940109568 19940621

Priority number(s): JP19940022895 19940221 ; JP19930227204 19930913

Also published as: EP0643140 (B1) US5670315 (A) DE69428855 (T2) CA2126391 (A1) CA2126391 (C) more

Abstract of EP0643140 (A1)

A PCR amplification product is detected, in quantitative determination of nucleic acid and measurement of the number of bacterial cells or specific genes, by addition of a dye compound which does not fluoresce in the free state but fluoresces in the bonded state to a double-stranded nucleic acid.

Last updated: 13.03.2013 Worldwide Database 5.8.11.1; 92p

Espacenet

Family list: EP0643140 (A1) — 1995-03-15

17 application(s) for: EP0643140 (A1)

1. Fluorescent stain containing pyrylium salt or its similar salt, detection method of nucleic acid by use of it, and fluorescent staining method of biological sample.					
Inventor: YAMAMOTO NOBUKO [JP] OKAMOTO TADASHI [JP] (+3)	**Applicant:** CANON KK [JP]	**CPC:** C09B57/00 C12Q1/6816 C12Q2304/10 (+3)	**IPC:** C07D309/34 C07D335/02 C07D345/00 (+10)	**Publication info:** AT206210 (T) 2001-10-15	**Priority date:** 1992-12-21
2. Determination of nucleic acid by PCR, measurement of number of microbial cells, genes, or gene-copies by PCR, and measuring-kit employed for the same.					
Inventor: YAMAMOTO NOBUKO [JP] OKAMOTO TADASHI [JP] (+4)	**Applicant:** CANON KK [JP]	**CPC:** C09B57/00 C12Q1/6851 G01N33/52	**IPC:** C09B57/00 C12Q1/68 G01N33/52 (+3)	**Publication info:** AT207966 (T) 2001-11-15	**Priority date:** 1993-09-13
3. Fluorescent stain containing pyrylium salt or its similar salt, detection method of nucleic acid by use of it, and fluorescent staining method of biological sample.					
Inventor: YAMAMOTO NOBUKO [JP] OKAMOTO TADASHI [JP] (+3)	**Applicant:** CANON KK [JP]	**CPC:** C09B57/00 C12Q1/6816 C12Q2304/10 (+3)	**IPC:** C07D309/34 C07D335/02 C07D345/00 (+10)	**Publication info:** AT343643 (T) 2006-11-15	**Priority date:** 1992-12-21
4. DETERMINATION OF NUCLEIC ACID BY PCR, MEASUREMENT OF NUMBER OF MICROBIAL CELLS, GENES, OR GENE-COPIES BY PCR, AND MEASURING-KIT EMPLOYED FOR THE SAME					
Inventor: YAMAMOTO NOBUKO [JP] OKAMOTO TADASHI [JP] (+4)	**Applicant:** CANON KK [JP]	**CPC:** C09B57/00 C12Q1/6851 G01N33/52	**IPC:** C09B57/00 C12Q1/68 G01N33/52 (+1)	**Publication info:** CA2126391 (A1) 1995-03-14 CA2126391 (C) 2002-01-08	**Priority date:** 1993-09-13
5. Fluorescent stain containing pyrylium salt or its similar salt, detection method of nucleic acid by use of it, and fluorescent staining method of biological sample.					
Inventor: YAMAMOTO NOBUKO [JP] OKAMOTO TADASHI [JP] (+3)	**Applicant:** CANON KK [JP]	**CPC:** C09B57/00 C12Q1/6816 C12Q2304/10 (+3)	**IPC:** C07D309/34 C07D335/02 C07D345/00 (+10)	**Publication info:** DE69330818 (T2) 2002-03-28	**Priority date:** 1992-12-21
6. Fluorescent stain containing pyrylium salt or its similar salt, detection method of nucleic acid by use of it, and fluorescent staining method of biological sample.					
Inventor: YAMAMOTO NOBUKO [JP] OKAMOTO TADASHI [JP] (+3)	**Applicant:** CANON KK [JP]	**CPC:** C09B57/00 C12Q1/6816 C12Q2304/10 (+3)	**IPC:** C07D309/34 C07D335/02 C07D345/00 (+8)	**Publication info:** DE69334079 (T2) 2007-05-16	**Priority date:** 1992-12-21

7. Determination of nucleic acid by PCR, measurement of number of microbial cells, genes, or gene-copies by PCR, and measuring-kit employed for the same.					
Inventor: YAMAMOTO NOBUKO [JP] OKAMOTO TADASHI [JP] (+4)	**Applicant:** CANON KK [JP]	**CPC:** C09B57/00 C12Q1/6851 G01N33/52	**IPC:** C09B57/00 C12Q1/68 G01N33/52 (+3)	**Publication info:** DE69428855 (T2) 2002-05-02	**Priority date:** 1993-09-13
8. Fluorescent stain containing pyrylium salt or its similar salt, detection method of nucleic acid by use of it, and fluorescent staining method of biological sample.					
Inventor: YAMAMOTO NOBUKO C O CANON KABU [JP] OKAMOTO TADASHI C O CANON KABU [JP] (+3)	**Applicant:** CANON KK [JP]	**CPC:** C09B57/00 C12Q1/6816 C12Q2304/10 (+3)	**IPC:** C07D309/34 C07D335/02 C07D345/00 (+10)	**Publication info:** EP0603783 (A1) 1994-06-29 EP0603783 (B1) 2001-09-26	**Priority date:** 1992-12-21
9. Determination of nucleic acid by PCR, measurement of number of microbial cells, genes, or gene-copies by PCR, and measuring-kit employed for the same.					
Inventor: YAMAMOTO NOBUKO C O CANON KABU [JP] OKAMOTO TADASHI C O CANON KABU [JP] (+4)	**Applicant:** CANON KK [JP]	**CPC:** C09B57/00 C12Q1/6851 G01N33/52	**IPC:** C09B57/00 C12Q1/68 G01N33/52 (+3)	**Publication info:** EP0643140 (A1) 1995-03-15 EP0643140 (B1) 2001-10-31	**Priority date:** 1993-09-13
10. Fluorescent stain containing pyrylium salt or its similar salt, detection method of nucleic acid by use of it					
Inventor: YAMAMOTO NOBUKO [JP] OKAMOTO TADASHI [JP] (+3)	**Applicant:** CANON KK [JP]	**CPC:** C09B57/00 C12Q1/6816 C12Q2304/10 (+3)	**IPC:** C07D309/34 C07D335/02 C07D345/00 (+10)	**Publication info:** EP1052291 (A2) 2000-11-15 EP1052291 (A3) 2000-11-22 EP1052291 (B1) 2006-10-25	**Priority date:** 1992-12-21
11. FLUORESCENT DYEING AGENT INCLUDING PIRILIUM SALT OR ANALOG OF PIRILIUM SALT AND DETECTION METHOD FOR NUCLEIC ACID USING IT AND FLUORESCENT DYEING METHOD FOR BIOLOGICAL SAMPLE					
Inventor: YAMAMOTO NOBUKO OKAMOTO HISASHI (+1)	**Applicant:** CANON KK	**CPC:** C09B57/00 C12Q1/6816 C12Q2304/10 (+3)	**IPC:** C07D309/34 C07D335/02 C07D345/00 (+15)	**Publication info:** JPH07174759 (A) 1995-07-14 JP3247001 (B2) 2002-01-15	**Priority date:** 1992-12-21
12. METHOD FOR DETERMINING NUCLEIC ACID BY PCR, METHOD FOR MEASURING NUMBER OF MICROORGANISM OR CELL, NUMBER OF SPECIFIC GENE AND NUMBER OF SPECIFIC GENE COPY BY PCR AND MEASURING KIT USED FOR THESE METHODS					
Inventor: YAMAMOTO NOBUKO OKAMOTO HISASHI (+4)	**Applicant:** CANON KK	**CPC:**	**IPC:** C12N15/09 C12Q1/68 G01N27/447 (+4)	**Publication info:** JPH07163399 (A) 1995-06-27 JP3683913 (B2) 2005-08-17	**Priority date:** 1993-03-10
13. FLUORESCENT DYE CONTAINING PYRYLIUM SALT OR PYRYLIUM ANALOGOUS SALT, AND DETECTION OF NUCLEIC ACID AND FLUORESCENT DYEING OF ORGANISM SPECIMEN USING THE SAME					

Inventor:	Applicant:	CPC:	IPC:	Publication info:	Priority date:
YAMAMOTO NOBUKO OKAMOTO HISASHI (+3)	CANON KK		C07D309/34 C07D335/02 C07D345/00 (+21)	JPH0940661 (A) 1997-02-10	1992-12-21
14. Fluorescent stain containing pyrylium salt or its similar salt detection method of nucleic acid by use of it and fluorescent staining method of biological sample					
Inventor:	Applicant:	CPC:	IPC:	Publication info:	Priority date:
KAWAGUCHI MASAHIRO YAMAMOTO NOBUKO (+3)	CANON KK	C09B57/00 C12Q1/6816 C12Q2304/10 (+3)	C07D309/34 C07D335/02 C07D345/00 (+10)	SG65577 (A1) 1999-06-22	1992-12-21
15. Detection method of nucleic acid by use of fluorescent pyrylium stain in intercalation into nucleic acids					
Inventor:	Applicant:	CPC:	IPC:	Publication info:	Priority date:
YAMAMOTO NOBUKO [JP] OKAMOTO TADASHI [JP] (+3)	CANON KK [JP]	C09B57/00 C12Q1/6816 C12Q2304/10 (+3)	C07D309/34 C07D335/02 C07D345/00 (+11)	US5624798 (A) 1997-04-29	1992-12-21
16. Nucleic acid determination employing pyrylium dye					
Inventor:	Applicant:	CPC:	IPC:	Publication info:	Priority date:
YAMAMOTO NOBUKO [JP] OKAMOTO TADASHI [JP] (+4)	CANON KK [JP]	C09B57/00 C12Q1/6851 G01N33/52	C09B57/00 C12Q1/68 G01N33/52 (+2)	US5670315 (A) 1997-09-23	1993-09-13
17. Fluorescent stain containing pyrylium salt and fluorescent staining method of biological sample					
Inventor:	Applicant:	CPC:	IPC:	Publication info:	Priority date:
YAMAMOTO NOBUKO [JP] OKAMOTO TADASHI [JP] (+3)	CANON KK [JP]	C09B57/00 C12Q1/6816 C12Q2304/10 (+3)	C07D309/34 C07D335/02 C07D345/00 (+10)	US6022961 (A) 2000-02-08	1992-12-21

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(54) **Determination of nucleic acid by PCR, measurement of number of microbial cells, genes, or gene-copies by PCR, and measuring-kit employed for the same.**

(57) A PCR amplification product is detected, in quantitative determination of nucleic acid and measurement of the number of bacterial cells or specific genes, by addition of a dye compound which does not fluoresce in the free state but fluoresces in the bonded state to a double-stranded nucleic acid.

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BACKGROUND OF THE INVENTION

Field of the Invention

The present invention relates to a method of determination of nucleic acid by detecting the polymerase chain reaction (PCR) amplification product by use of a dye compound which does not fluoresce in the free state but fluoresces reacting with double-stranded nucleic acid. The present invention also relates to a method for measuring the number of microbial cells, the number of specified genes and the number of specified gene copies. The present invention further relates to a measuring-kit to be utilized for the above method. The method is useful for measuring the number of cells of a specified microorganism in a solution or in soil.

Related Background Art

The PCR is a method for enzymatically amplifying a specific DNA sequence by using the specified sequence as a template defined with two kinds of primers. This method has become utilized in detection of nucleic acid. Detection is conducted by steps of selecting a specific sequence of the target nucleic acid, preparing a set of primers for amplifying a specific sequence, conducting PCR by utilizing the target nucleic acid as a template, and detecting the amplified specific sequence. According to PCR, the specific sequence which is characteristic of the object to be detected is amplified with a high amplification rate, enabling the detection of even a minute amount of target nucleic acid in a sample. For example, the reaction for several hours can give about 1,000,000-fold amplification. Therefore, even one molecule of nucleic acid can be detected by PCR. This amplification proceeds only when the two primer sequences have complementarity to the template nucleic acid, and no amplification product can be obtained when the complementary sequence is not present.

Thus, PCR, which greatly improves the sensitivity of nucleic acid detection, has come to be utilized to detect nucleic acid in various technical fields in place of the hybridization method: particularly, to identify the pathogen of a virus- or bacterial disease in clinical tests, to analyze genes in genetic disorders, and to detect gene markers in cancer diagnosis.

Another application of PCR is in measurement of the cell number of a certain microorganism, which is conducted with combination of PCR with MPN (most probable number) method, [H.O. Halvorson, and N.R. Ziegler: J. Bacteriol., 25 101 (1933)]. In MPN method, which is also called the dilution count method, a sample is serially diluted, a predetermined portion of each sample dilution is inoculated into a culture medium in a test tube, and incubated for a sufficient period, thereafter occurrence of cell growth is observed for each tube, and the statistic treatment of the result gives the most probable cell number of the specimen ("Dojobiseibutsu Jikkenho (Experiment in soil microbiology)", page 45, published by Yokendo Co.). This method has disadvantages that the procedure is troublesome and requires many test tools and long incubation period especially for soil microorganism determination. Moreover, by MPN method, the number of the specific microbial cells cannot be determined when two or more kinds of microorganisms are present in the sample, since the MPN method is positive for all the microorganisms grown in the employed culture medium. To offset such disadvantages, trials have been made to detect a microorganism by the DNA level using DNA amplification. One method therefor is a combination of PCR with MPN. In this PCR-MPN combination method, DNA is extracted from a sample containing the target microorganism, the extracted DNA is serially diluted, the dilutions are subjected to PCR with two kinds of primers to amplify the nucleic acid sequence characteristic of the target microorganism, and the number of the target microbial cells is determined by detecting the amplification product.

PCR amplification gives the product DNA in an amount of micrograms from picograms of starting DNA in a short time, which enables rapid in vitro amplification of cloned DNA or genome DNA in a large amount. Thus, the PCR is now being applied to cloning and various detections. In PCR, the target double-stranded DNA is denaturated by heating to give single-stranded DNA, and the primers anneal to each of the single-stranded DNA which serves as the template, and a complementary strand is synthesized by extending the primer with DNA polymerase. Therefore, theoretically, one strand of template DNA is required for amplification. When genome DNA is employed as a template DNA, the length of the single-stranded template DNA greatly varies depending on the kind of the source organism. For example, the human genome DNA has a length of 10^3 times that of Escherichia coli. Thus, the number of DNA strands in one picogram differs by several numerical orders depending on the kind of the source organism.

The MPN-PCR method detects the number of DNA based on the above quantitative characteristics. In this method, the target DNA is serially diluted to the extent that a certain dilution provides only one

molecule of DNA or none in the reaction system, and then the DNA is amplified and detected to determined the number of the template DNA molecule in the original sample by probability technique. Accordingly, this MPN-PCR method utilizes the principle of the MPN method of measurement of microbial cell number replacing the microorganism in MPN method with the template DNA, and the growth of the microorganism with PCR. In other words, in MPN, a sample containing microbial cells is serially diluted to such a concentration that one cell is present or not present in a certain volume of a dilution, then incubated for cell proliferation detection. The number of the cells in the starting sample is estimated from the results using probability technique. In the MPN-PCR method, the microorganism is replaced with template DNA, and the incubation process with PCR. Accordingly, in the MPN-PCR method, the object of the measurement is not the quantity of the target DNA, but the number of DNA molecules.

An example of the conventional MPN-PCR method is described specifically below. DNA extracted from a microorganism-containing sample is serially diluted to prepare decimal dilutions (e.g., dilution rate of 1, 10^{-1}, 10^{-2}, ..., 10^{-9}). Here, the dilution rate 10^{-n} means that the sample is diluted to contain $1/10^n$ of the original sample. The respective dilutions (10 dilutions in this case) are subjected to PCR to amplify the target DNA. After the PCR, the reaction solution is subjected to agarose gel electrophoresis to detect the amplification product as an electrophoresis band. In a series of the dilutions, the band of the amplification product becomes not detectable at a certain dilution and thereafter. For example, if the amplification product is detected at the dilution rate of 10^{-5} and dilutions lower than that, PCR is carried out for the highest dilution in which the product was detected and two adjacent dilutions, namely the dilutions of 10^{-4}, 10^{-5}, and 10^{-6}, in quintuplicate (15 samples in total), followed by agarose gel electrophoresis to detect the amplification product. The number of the samples positive for the amplification product in quintuplicate at the respective dilution is compared with the MPN table shown later (cited from J. Bacteriol., 25 101 (1933), page 400), from which the most probable number is obtained. In this example, if 5 reactions of 10^{-4} dilution are all positive (namely, amplification product being detected), 3 out of 5 reactions of the 10^{-5} dilution are positive, and 1 out of 5 reactions of the 10^{-6} dilution is positive, from the numerals of 5, 3, and 1, a value (1.1) is obtained by applying 5, 3, and 1 to P_1, P_2, and P_3 in the MPN table. This value (1.1) multiplied by the reciprocal of 10^{-5} which is the highest dilution detected for the PCR amplification product, namely 1.1×10^5, is the number of the target DNA in the original sample DNA.

Assuming that one molecule of the target template DNA exists in one cell, the number of the template DNA measured by the MPN-PCR method is equal to the number of the cells in the sample from which the sample DNA was prepared. When the target gene belongs to a genome, however, several copies may exist for one gene. In such a case, it is necessary to determine the correlation between the cell number and the number of the DNA preliminarily since the number of DNA does not directly correspond to the cell number. Furthermore, the DNA extraction efficiency also needs to be taken into account. Therefore, for a precise measurement result, it is desirable to draw a calibration curve. This method is also applicable to the quantitative determination of the cells having plural copies of a specific gene due to the amplification as in the cancer cell.

The amplification product of PCR is conventionally detected, for example, by developing the PCR reaction mixture by gel electrophoresis to separate the amplification product from the other components such as the template nucleic acid and the primers, identifying the fluorescence- stained band of the amplification product in consideration of its molecular weight, and measuring the fluorescence intensity.

The PCR reaction mixture contains also the template nucleic acid, excess amounts of primers, etc. Therefore, the separation of the amplification product from other non-target components by gel electrophoresis becomes difficult depending on the nature of the nucleotide sequence of the target DNA. Moreover, when there are many test samples, it is laborious and time-consuming to carry out complicated electrophoresis, which lowers the efficiency of the detection operation. Therefore, the conventional techniques are not sufficient enough for especially gene analysis in the clinical examination where many samples should be treated with high efficiency.

In the application of PCR to MPN method, in order to estimate the precise number of the microbial cells, the samples are serially diluted by ten-fold, each dilution is PCR amplified, and the experiment is repeated for three serial decimal dilutions including the highest dilution in which the amplification product has been detected. Since this method estimates the cell number based on probability, usually at least 5 to 10 repetition for each dilution level are required to meet the conditions for the estimation of the cell number on the basis of probability from the MPN table. Accordingly, at least about 25 PCR products for one sample should be subjected to gel electrophoresis, which means a lot of labor, complicated operations, and time to obtain the result.

As mentioned above, detection of nucleic acid or measurement of microbial cell number by PCR requires, in many cases, complicated and time-consuming operation to detect or determine the amplifica-

tion products after the amplification reactions, hindering efficient treatment of a large number of samples. Therefore, a simple and precise method is strongly desired for detection and determination of PCR amplification products.

The most troublesome operation in detecting PCR amplification product is the gel electrophoresis to separate the amplification product from the primers added to the reaction solution in large excess. Various methods are investigated to omit the separation operation. Of the methods, noteworthy is a method employing a fluorescent intercalating agent (an intercalator) which increases fluorescence intensity when bound to double-stranded nucleic acid. In this method, the fluorescent intercalator reacts with the double-stranded nucleic acid which has been amplified by PCR, and the increase of fluorescence intensity caused by the reaction is measured to detect the amplification product. Theoretically, in this method the primer (single-stranded DNA) does not increase the fluorescence intensity of the intercalator. Therefore, this method advantageously saves the troublesome separation operation.

Japanese Patent Application Laid-Open No. 5-237000, for example, discloses use of a dye, as the fluorescent intercalator, such as ethidium bromide, acridine orange, bisbenzimide, diaminophenylindole, actinomycin, thiazole orange, chromomycin, and derivatives thereof for detection of the PCR amplification product. Of these dyes, ethidium bromide is preferred of which fluorescence intensity increases as much as about 50-fold under UV excitation light when bound to double-stranded nucleic acid, and about 20-fold under visible excitation light, in comparison with that in the free state (before the reaction with the double-stranded nucleic acid).

However, the dyes mentioned in the above Japanese Patent Application Laid-Open No. 5-237000 have fluorescence even in the free state. The measured fluorescence thus includes both the fluorescence produced by the double-stranded nucleic acid-dye complex and that of the free dye. Therefore, the fluorescence of the free dye should be subtracted as the blank value from the measured value. In other words, in this method, the measured value does not give directly the presence or the quantity of the amplification product, and the blank value should be subtracted therefrom. When the amount of the amplification product is small, the increase of fluorescence intensity from the blank value may be small, and the sensitivity of the detection becomes inevitably low.

YOYO-1 (Nucleic Acids Research, 20 (11), 2803-2812 (1992)) emit little fluorescence in the free state, but when intercalated into double stranded DNA, a large increase of fluorescence intensity (about 3000-fold) will occur. With this dye, the above disadvantage of a high blank value can be offset. This dye, however, is not practically useful because it decomposes at room temperature. Moreover, this dye also detects the higher-order structure formed between the primers, as mentioned later, which disadvantageously prevents precise quantitative determination.

From the above viewpoint, in the above Japanese Patent Application Laid-Open No. 5-237000, the amplification cycle is continued till a sufficient fluorescent intensity is obtained in comparison with the blank value: the number of necessary cycles is in reverse proportion to the initial concentration of the target nucleic acid. In the determination of a target nucleic acid in the specification of the above Patent Application, the change of the fluorescence intensity is monitored in the course of the PCR amplification, and the initial concentration of the target nucleic acid is determined from the cycle number at which the fluorescence intensity changed abruptly.

Such a method of determination requires a troublesome operation of monitoring the fluorescence intensity in each cycle of the PCR reaction. Moreover, it is sometimes difficult to detect the point of the significant change of the fluorescence intensity. Therefore, the method still has problems in efficiency and sensitivity for quantitative determination of the target nucleic acid.

When the PCR amplification product is detected with ethidium bromide (EB) or the like, it is theoretically possible to detect the amplification product in the reaction mixture in the presence of the primer set, since the fluorescence intensity does not increase on reaction with primers. However, according to the studies by the inventors of the present invention, primers react each other during the PCR to form aggregates (higher order structures having a three-dimensional structure) which is also detected with ethidium bromide or the like. If the amplification product is formed in a much larger amount than the higher-order structured primers, no problems arises. If not, the ratio of the fluorescence due to the higher-order structure matter in the measured fluorescence becomes larger, which prevents precise quantitative determination. The amount of the higher-order structured matter is not steady since it becomes small when the amplification products is formed in overwhelming amount, while it tends to be formed more when a little or no amplification product is formed. Therefore, the correction by comparison with the blank value is not easy.

The formation of the higher-order structured matter can be confirmed, for example, by conducting PCR in the absence of template nucleic acid, developing the reaction mixture by gel electrophoresis, and

observing a cloud-like pattern stained with ethidium bromide in the low molecular weight zone.

The measurement error caused by the higher-order structure is ascribable to the PCR itself, and is liable to be more significant when the amplification is conducted for more cycles with a lower initial concentration of the target nucleic acid.

Accordingly, the conventional methods of determination of amplification product as described above are still insufficient for higher sensitivity.

The measurement of microbial cells by combination of MPN with PCR also involves the same problem of formation of the higher-ordered structure matter from the primers, inviting large determination error.

SUMMARY OF THE INVENTION

The present invention intends to provide a method for precise determination of a PCR amplification product in a simple operation.

The present invention also intends to provide a method for measuring the number of target microorganism or cells, the number of a specified gene, or the copy number of a specified gene.

The present invention also intends to provide a measuring kit for the above methods.

The method to determine the nucleic acid quantity of the present invention comprises conducting PCR on the sample nucleic acid using a primer set which is necessary to amplify a specified sequence region of the target nucleic acid, then reacting the double-stranded amplification product which is formed when the target nucleic acid is present in the sample, with a dye compound which does not fluoresce in the free state but fluoresces when reacted with the double-stranded nucleic acid, followed by measuring the fluorescence intensity to determine the quantity of the target nucleic acid in the sample.

The measuring kit for the nucleic acid determination of the present invention comprises a reactor in which a necessary amount of a dye compound which does not fluoresce in the free state but fluoresces when reacted with double-stranded nucleic acid is placed in a PCR reaction chamber. This measuring kit may contain in the reaction chamber a necessary amount of a primer set required for PCR to amplify a specified sequence region of the target nucleic acid. The measuring kit may have a PCR reaction chamber and separated from it, a reagent chamber wherein a necessary amount of a dye compound which does not fluoresce in the free state but fluoresces when reacted with double-stranded nucleic acid is placed so as to be fed to the reaction chamber. This kit also may contain in the reaction chamber a necessary amount of a primer set required for PCR amplification of the specified sequence region of the target nucleic acid.

The method for measuring the number of a target microorganism or cells, the number of a specific gene, or the copy number of a specific gene comprises extracting the nucleic acid from a sample containing a microorganism or cells to be detected, preparing serial dilutions of the extracted nucleic acid, conducting PCR on the diluted samples to amplify a sequence which is characteristic of the microorganism or the cells, reacting the resulting amplified double-stranded product with a dye compound which does not fluoresce in the free state but fluoresces in the bonded state to the double-stranded nucleic acid, measuring the intensity of the fluorescence, and deriving the number of the microorganism or the cells, the specified genes, or copies of the specified gene from the dilution where the fluorescence was observed.

The measuring kit used for the above measurement comprises a reactor having a plurality of reaction chambers for PCR and containing therein a required amount of a dye compound which does not fluoresce in the free state but fluoresces when in the bonded state to double-stranded nucleic acid, the reaction chambers can be employed for serial dilution of nucleic acid sample extracted from a sample containing target microorganism or cells, as well as the PCR of each dilution for amplification of a sequence which is characteristic of the target microorganism or cells. The each reaction chamber of the kit may further contain a primer set for PCR of the sequence characteristic of the target microorganism or cells.

The present invention mentioned above is applicable also to an MPN-PCR method as described later.

BRIEF DESCRIPTION OF THE DRAWINGS

Fig. 1 illustrates an arrangement of samples prepared in Example 1 on a microplate.

Fig. 2 shows the relation between the fluorescence intensity and the amount of template DNA in Example 1.

Fig. 3 shows the relation between the fluorescence intensity and the amount of template DNA in Comparative Example 2.

Fig. 4 illustrates an arrangement of samples prepared in Example 2 on a microplate.

Fig. 5 shows the formation of a PCR amplification product in the respective samples on the microplate in Example 2. The shadowing shows the wells of which fluorescence intensity was twice or more of the

blank.

Fig. 6 shows correlation between the cell numbers obtained in Example 2 and those obtained in Comparative Example 3.

Fig. 7 shows the position of the wells of which fluorescence intensity were twice or more that of the blank value in Example 3.

Fig. 8 shows the results of Example 4 with the sample from the tissue A.

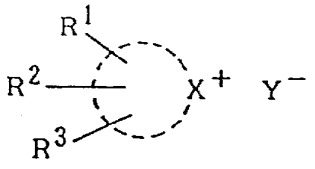
Figs. 9A to 9C show the construction and the method of use of a nucleic acid-detecting kit of the present invention.

DETAILED DESCRIPTION OF THE PREFERRED EMBODIMENTS

The dye compound employed in the present invention does not fluoresces in the free state but fluoresces when reacted with double-stranded nucleic acid. Reaction of the dye compound with double-stranded nucleic acid means specific insertion of the dye compound into grooves of a stable double helix structure of double-stranded nucleic acid to bind thereto. In that state, the dye compound fluoresces on irradiation of excitation light. The dye compound does not fluoresces in the free state, i.e., in the separated state, on irradiation of excitation light.

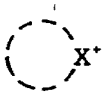
This dye compound does not readily enter short double-stranded portions of primers which do not form stable double helix structure. Even if it enters that portion, it produces no fluorescence, that is, it fluoresces only in the bonded state to the stable double helix structure. Accordingly, the dye compound of the present invention is the one which enters a main groove or a sub groove of a double helix structure and links thereto, producing fluorescence in that state. The use of a dye compound having such characteristics enables precise and selective detection of the amplification product by simply adding directly the dye compound to a PCR reaction mixture without separating the amplification product from the primers and template nucleic acid. The fluorescence of the dye compound in the free state may be negligibly weak.

The dye compound is exemplified by DAPI (4',6-diamino-2-phenylindole dihydrochloride), Hoechst 33258 (trade name), Hoechst 33342 (trade name), pyrylium compound salts and other salts analogous thereto represented by the general formula [I] below:



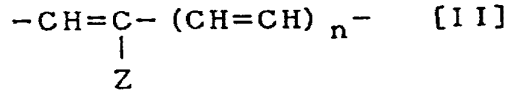
[I]

where



represents a heterocycle, and X is O, S, Se, or Te, the heterocycle including a five-membered and six-membered ring such as a pyrylium ring and pyrylium-analogous ring; R^1 and R^2 are independently a hydrogen atom, a halogen atom, a sulfonate group, an amino group, a styryl group, a nitro group, a hydroxyl group, a carboxyl group, a cyano group, a substituted or unsubstituted lower alkyl group, a substituted or unsubstituted aryl group, a substituted or unsubstituted lower aralkyl group, or a substituted or unsubstituted cycloalkyl group; R^3 is a group of -A or - L-A wherein L is $-L^1-$, $-L^2-L^3-$, or $-L^4-L^5-L^6-$, L^1 to L^6 being independently -(CH = CH)-, a bivalent group derived from a substituted or unsubstituted aryl group, a substituted or unsubstituted alkylene group, or $-CH=R^4-$(R^4 representing a cyclic structure having an oxo group). The bivalent group derived from a substituted or unsubstituted aryl group includes a phenylene group and the like, and may have the bonds in any of ortho, meta, and para positions. The lower alkylene group includes linear or branched alkylene group having 1 to 4 carbons, the substituent including groups of -L-A. The cyclic structure having an oxo group includes a heterocycle, aromatic ring, and aliphatic ring having at least an oxo group.

The group -L- preferably includes those represented by the general formulas [II], [III], [IV], [V], and [VI] below:



[II]

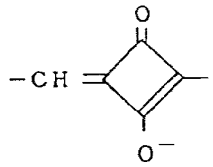
where Z is a hydrogen atom, or a substituted or unsubstituted lower alkyl group; and n is 0, 1, or 2, and the substituent for the alkyl group is exemplified by -L-A defined above;

$-\Phi-(CH=CH)_n-$ [III]

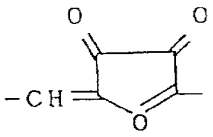
where n is 0, 1, or 2, and Φ is a substituted or unsubstituted o-, m,- or p-phenylene.

$-CH=CH-\Phi-CH=CH-$ [IV]

where Φ is a substituted or unsubstituted o-, m-, or p-phenylene group;



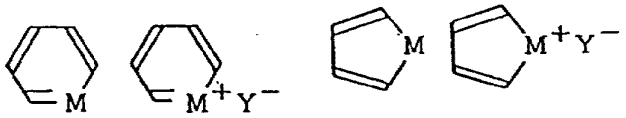
[V]

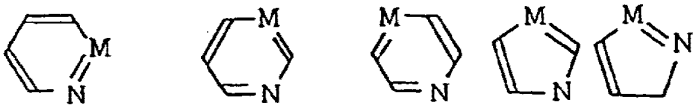


[VI]

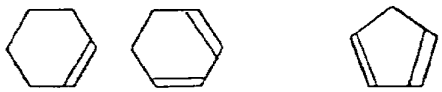
In the above general formulas, the substituent of the phenylene includes the groups mentioned above.

The group A in R^3 in General Formula [I] is a substituted or unsubstituted aryl, or $-CH=R^5$ (R^5 being a substituted or unsubstituted heterocycle, a substituted or unsubstituted cycloalkyl, or a substituted or unsubstituted aromatic ring). The heterocycle for R^5 includes the ones derived from the groups shown below:





where M and N are independently an oxygen atom, a sulfur atom, or a nitrogen atom, and Y^- is an anion. The substituent therefor includes a substituted or unsubstituted aryl group. The substituted or unsubstituted cycloalkyl group may be saturated or unsaturated and includes the ones derived from the groups below capable of constituting a resonance system:



The substituted or unsubstituted aromatic ring includes an azulene ring. The substituent linked to the above groups includes lower alkyl groups, and substituted or unsubstituted aryl groups.

In the pyrylium ring or an analogous ring containing X, the hydrogen atom bonded to the carbon atom which is not linked to R^1, R^2, or R^3 may be substituted by a halogen atom, a sulfonate group, an amino group, a styryl group, a nitro group, a hydroxyl group, a carboxyl group, a cyano group, a substituted or unsubstituted lower alkyl group, a substituted or unsubstituted aryl group, or a substituted or unsubstituted lower aralkyl group.

Y^- is an anion, including BF_4^-, perchlorate ion, $HO_3SCH_2COO^-$, halide ion such as chloride ion, bromide ion, iodide ion, and fluoride ion, a compound functioning as an anion such as aliphatic sulfonates and aromatic sulfonates, and complex ions of transition metals such as Zn, Ni, Cu, Pt, Co, and Pd.

When the above substituent is further substituted by a halogen, the halogen includes Cl, Br, and I. The lower alkyl group may be linear or branched, and is preferably of 1 to 4 carbons. The aryl group includes a phenyl group or the like. The substituent of the aryl or phenylene group includes an amino group substituted with a lower alkyl group (lower-alkylamino group). Such a lower-alkyl amino group include preferably dimethylamino, diethylamino, or the like at a para position. The lower aralkyl group includes lower alkyl groups substituted by the aforementioned substituted or unsubstituted aryl.

Of the compounds represented by General Formula [I], the X-containing heterocycle is preferably substituted by two or more substituted or unsubstituted aryl groups. The examples of such compounds having six-membered heterocycle are:

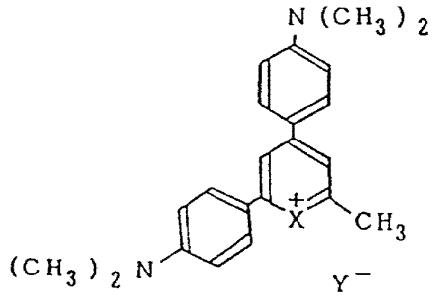
(1) those having substituted or unsubstituted aryl groups at 2- and 4-positions and an R^3 group at any one of 3-, 5-, and 6-positions of the X-containing six-membered ring;

(2) those having substituted or unsubstituted aryl groups at 3- and 5-positions and an R^3 group at any one of 2-, 4-, and 6-positions of the X-containing six-membered ring; and

(3) those having substituted or unsubstituted aryl groups at 2- and 6-positions and an R^3 group at any one of 3-, 4-, and 5-positions of the X-containing six-membered ring.

The introduction of substituted or unsubstituted aryl groups to such positions is preferred to obtain satisfactory properties as an intercalator in a nucleic acid base pairs. Additionally, the X-containing heterocycle is preferably substituted by two or more substituted or unsubstituted aryl groups such that the substituting positions are not adjacent to each other.

Specific examples of the compound of General Formula [I] are shown later in Table 1. Of these, particularly preferred are 2,4-bis(N,N,-dimethylaminophenyl)-6-methylpyrylium salts and 2,4-bis(N,N,-dimethylaminophenyl)-6-methylthiopyrylium salts represented by General Formula [VII]:



[VII]

where X is O or S, and Y^- is an anion. These compounds are preferred because of its very high intensity of fluorescence in the inserted state into double-stranded nucleic acid.

The dye compound employed in the present invention reacts selectively with the stable double-stranded helix DNA to produce fluorescence. Therefore, even if the dye compound is incorporated in a higher-order structure formed by the aforementioned reaction from primers, the compound does not fluoresce because the double-stranded portion of the higher-order structure is not a stable double-stranded structure.

The compound of General Formula [I] is introduced specifically into the double-stranded structure in a ratio of one molecule to 20 to 30 base pairs, the compound is advantageously not liable to be inserted into the higher-order structure derived from primers as mentioned above. Further, when the compound of General Formula [I] is incorporated into a double-stranded nucleic acid segment having a sequence to be amplified, the incorporation density is much lower than that of ethidium bromide or the like, so that the elevation of melting temperature (Tm) of the double-stranded nucleic acid hardly occurs not affecting the PCR. Because of the low incorporation density, when the PCR product is analyzed by gel electrophoresis, the mobility of the double-stranded nucleic acid is hardly affected by the incorporated dye compound, thus enabling precise size measurement.

On the contrary, a conventionally used fluorescent intercalator such as ethidium bromide, acridine orange, and YOYO-1 is inserted into the double-stranded nucleic acid at a ratio of one molecule to several base pairs (about 2 to about 5 base pairs). These dyes are incorporated into the double-strand portion of the aforementioned higher-order structure formed from the primers, which causes increase of undesired fluorescence. Also these dyes greatly affect Tm of the double-stranded nucleic acid and the mobility thereof in gel electrophoresis because of the high incorporation rate into the target double-stranded nucleic acid.

To conduct precise quantitative determination by utilizing the above benefit of the dye compounds in the present invention, the amplification product is desired to form a stable double helix structure. Therefore the present invention is useful when the amplification products have a length of 100 base pairs or more, for example, preferably 300 base pairs. The maximum length of the amplification product is not particularly limited. The velocity of incorporation of the nucleotide in PCR is said to 35 to 100 nucleotides per second, for example, at 72 °C. The rate depends on the reaction conditions such as pH and salt concentration of the reaction medium, and the base sequence of the target nucleic acid. Accordingly, when the reaction time of each cycle of PCR is one minutes, the length is preferably not more than 2000 base pairs. In addition, since byproducts tend to be formed when the chain length is excessively long, it is preferable that the length is not more than 1000 base pairs ("PCR Protocols", edited by Michael A. Innis, David H. Gelfand, John J. Sninsky, and Thomas J. White, 1990, (Academic Press Inc., San Diego, Calfornia 92101)).

In the PCR, a primer set which is capable of defining the intended specific region of the target nucleic acid is used according to the purpose. The primer is required to have a sufficient length for recognizing the specific sequence at the end of the specific region to be amplified of the template nucleic acid, but should not be unnecessarily long, since excessively long primers tend to form locally a double-stranded structure between the primers. Therefore, the length of the primer is less than about 30 base pairs, preferably less than about 28 base pairs. The minimum of the primer length is not specially limited, but is required to have a sufficient length for recognizing the terminal portion of the specific region to be amplified: e.g., not less than 14 base pairs, preferably not less than 18 base pairs.

In the quantitative determination of nucleic acid of the present invention, firstly a sequence region is specified which is characteristic of the target nucleic acid to be determined. Secondly, PCR is conducted using a primer set necessary for amplification of the specified sequence region and the target nucleic acid as the template. Subsequently, the resulting amplified product is reacted with a dye compound having the aforementioned properties, and the intensity of the fluorescence is measured with irradiation of excitation light. The intensity of the fluorescence is proportional to the initial concentration of the template (the concentration before the PCR) provided that the concentrations of the reaction components are selected suitably. Therefore, the target nucleic acid in an unknown sample can be determined by conducting PCR and then measuring the fluorescence intensity by reference to a calibration curve prepared preliminarily for the fluorescence intensity as a function of the template concentration.

The quantitative determination can be conducted simply by use of a calibration curve as mentioned above with a reaction system for which the optimum condition has been established. The reaction conditions, however, especially the amount of the target nucleic acid, the amount of the primers, the reaction temperature, etc. depend on the respective reaction systems. Therefore, when the optimum reaction conditions for calibration curve preparation are unknown, complicated operations are necessary for establishing the conditions.

When the conditions for calibration curve preparation are not established in the system, the approximate quantity of the target nucleic acid can be measured by simply preparing serial dilutions of the nucleic acid of an unknown sample, conducting PCR with the diluted samples, and determining the target nucleic acid segment by use of an MPN-PCR method which gives probability of detection of the target nucleic acid segments. Thereby the conditions for preparing the calibration curve can be obtained easily. For instance, in the specific example described before in the Related Background Art, the number of the template DNA in the sample is 1.1×10^5, and at 10^{-5} dilution, three samples out of the five samples are positive. In this case, at the concentrations of 10^{-4} dilution or higher, all of the five tests of each concentration will be

positive. In this example, firstly the concentration range including the critical dilution of the template DNA is determined according to the presence or absence of the amplification product, and then detailed MPN investigation is conducted on the concentration region. This is because, in agarose gel electrophoresis, the detection is conducted in an ON/OFF manner, the presence or absence of the band, and even when the DNA is quantitatively detected by fluorescence staining, the quantitation is not sufficient in spite of the complicated operations. On the other hand, the method of the present invention employing an MPN-PCR method as described later makes it practicable to check the formation of amplification product for all over the dilutions, by use of a tool such as a microplate which can provide many reaction zones (chambers). For instance, when the sample described in the Related Background Art is treated on a microplate according to the present invention, positive wells and negative wells will be observed at the 10^{-5} dilution, while at the lower dilution levels, 10^{-4} dilution or lower, the same intensity of fluorescence will be observed in the same dilution level, because the amplification proceeds in the same degree in the same dilution level. The plots of the average fluorescence intensity of the five reaction of respective dilution as a function of dilution levels will give a straight line. The longer the straight line region, the more appropriate is the PCR conditions. If the plots deviate significantly from the straight line, it means that the quantity of the primer or other conditions need to be changed.

If the fluorescence intensity is in a linear relation with the dilutions, the number of the template DNA and the quantity thereof (number × molecular weight) can be determined quantitatively from this calibration line since the number of the template DNA is known in this MPN-PCR.

As described above, the method of the present invention enables direct and selective measurement of the fluorescence which is produced only when the amplified product reacted with a dye compound added to the PCR mixture. Therefore, the separation of the amplified product from the primer and the template is unnecessary and even when the higher-order structure are formed between the primers, the influence of it on the measurement result is negligible. Accordingly, the fluorescence intensity obtained by directly adding the dye compound to the reaction mixture can be used for calculation of the precise quantitative determination. When ethidium bromide or a similar substance which fluoresces even in the free state is used for the quantitative determination, the blank value has to be subtracted from the measured value. Since the blank value varies depending on the measuring conditions, the determination conditions have to be decided in consideration of the relation of the blank value to the measured value for each of measurement conditions, which is quite troublesome. In the method of the present invention, such blank value consideration or a set up of determination conditions is not necessary, differing from with ethidium bromide, since the dye compound of the present invention produces no or negligible fluorescence in the free state. Further in the present invention, the fluorescence intensity need not be monitored during the PCR process as described in Japanese Patent Application Laid-Open No. 5-237000.

Furthermore in the present invention, a microplate may conveniently be used for the detection by transferring the PCR reaction mixture from the reaction vessel to the microplate well and measuring the fluorescence by use of a microplate fluorescence detector (reader), conveniently requiring a small amount of a reagent and a short measurement time. The PCR process itself may be conducted on the microplate, which more simplifies the determination operation.

A nucleic acid determining kit can be provided by employing a reactor like a microplate having many wells as reaction chambers and placing a necessary amount of a dye compound of the present invention by applying a solution thereof to each of the reaction chambers, followed by evaporation or drying or the like. Such a constitution is practicable owing to the stability of the dye compound at room temperature.

Another type of kit is also useful in which reagent chambers are provided separately from the PCR reaction chambers, and the dye compound of the present invention placed in the reagent chambers is added to the reaction chambers in the PCR step. The dye compound may be placed in the reagent chamber as a solution. If the dye compound is water-soluble, it may be solubilized in a suitable buffer solution, and if the compound is soluble in an organic solvent, the compound may be placed as a solvent solution.

The reagent chamber may contain a buffer solution, nucleotides, the enzyme, etc. necessary for the PCR together with the dye compound. When all of these PCR substances are dissolved in an aqueous solution, the organic solvent for the water-insoluble dye compound is preferably not more than 1 % based on the aqueous solution. This constitution of placing the reagents for PCR and the dye compound together in a reagent chamber is possible because the dye compound of the present invention is incorporated into the double-stranded nucleic acid segments (amplification product) at much lower density than ethidium bromide does, resulting in little elevation of the melting temperature (Tm), thus hardly affecting PCR reaction efficiency.

Figs. 9A to 9C show an example of constitution of the kit having such a reactor. In an appropriate container 1 which forms a reaction chamber 5, primer 3 is placed by liquid application or a like method, and further a container 2 is placed in it as the reagent chamber which is made of paraffin-coated paper or the like to be liquid-tight, and in which packed is a solution containing the enzyme, nucleotides, a buffer solution, etc in an amount necessary for the PCR (see Fig. 9A and Fig. 9B). With this device, PCR is conducted by injecting a solution of template nucleic acid into the reaction chamber 5 by a suitable means such as a Pipetman, concomitantly breaking through the container 2 to feed the contents therein to the reaction chamber 5, and mixing the reaction components to start PCR (see Fig. 9C). The dye compound for detection of the amplified product may be added after the PCR, or may be placed preliminarily in the reaction chamber. Otherwise, the dye compound may be contained in the solution in the reagent chamber 2.

Two or more reagent chambers may be provided for storing separately the reaction components.

The dye compound may be added to the reaction chamber for PCR prior to or after the PCR. In the latter case, another reagent chamber may be provided in which a necessary amount of the dye compound is held, and the dye compound may be added to the reaction chamber after the PCR from the reagent chamber. Of the compound represented by General Formula [I], water-insoluble ones remain undissolved, even if it is placed in the reaction chamber during the PCR, and the compound is allowed to react after the PCR by addition of a suitable solvent to the reaction chamber. The water-insolubility of the compound eliminates the undesirable influence of the compound on PCR, even though in general such effect is inherently small for the compounds of General Formula [I]. The suitable solvent used therefor includes acetonitrile, ethanol, dimethylsulfoxide (DMSO), etc. The operation of PCR in the reaction chamber can be simplified by placing the primer separately and preliminarily in the reactor by liquid application or in a powder state.

The detection of the amplification product of PCR with the dye compound of the present invention is suitably conducted by MPN utilizing PCR (MPN-PCR method). This method enables measurement of the number of individuals of microorganisms, animal cells, human cells, vegetable cells, etc. (the number of cells, the number of bacteria, the number of mycelia, etc.), and the number of specific genes or the number of copies of the specific gene of the above individuals with greatly simplified operations.

An example of the MPN-PCR method is described below for measurement of the number of individuals of a microorganism (cell number).

A soil sample is treated to extract the nucleic acid of microorganisms in soil. For example, to a soil sample (1 g), a phosphate buffer solution (1 ml) is added, and the mixture is agitated by a vortex mixer twice for 20 seconds each. Thereto 1/10 volume of 10% SDS was added, and the mixture is voltexed again. The resulting mixture is kept at 70 °C for one hour for cell lysis. Then the soil is removed by centrifugation to collect the supernatant as the nucleic acid fraction. Thereto, 1/5 volume of 7.5M sodium acetate is added, and the mixture is left standing at 4 °C for 5 minutes. The supernatant (1 ml) is recovered, and thereto, isopropanol (4/5 volume) is added. The nucleic acid fraction is recovered by centrifugation, and dissolved in a TE buffer solution (0.1 ml). DNA is recovered therefrom after RNase treatment.

An aliquot of the recovered DNA is diluted 100-fold. Starting from it, a series of dilutions was prepared to make 10^{-1} to 10^{-8} dilutions. These dilutions are placed in the sample block A on the microplate. More specifically, the 10^{-1}-dilution of the starting DNA solution (100-fold diluted soil extract), is placed in A1 to A5 wells, the 10^{-2}-dilution solution at the wells B1 to B5, and so forth, and 10^{-8}-dilution solution at the wells H1 to H5. Then a reaction solution containing reagents (a nucleotide, a primer salt, a Taq polymerase, etc.) necessary for PCR are added to all the wells including the blanks to allow the PCR to proceed. After the reaction, the fluorescence intensity of respective wells are measured by use of a dye compound. For example, the well which fluoresces at an intensity of twice or more that of the blank value is defined to be positive, and the dilution limit for the positive fluorescence is derived. If the numbers of positive samples out of the 5 samples at each of the dilution level (positive sample number/total sample number) are 5/5 at 10^{-4} dilution, 3/5 at 10^{-5} dilution, and 0/5 at 10^{-6} dilution, the critical dilution for the positive fluorescence is 10^{-5}. The numbers of the positive samples (5, 3, and 0) at these dilution degrees are respectively applied to P_1, P_2, and P_3 shown in the MPN table shown later (cited partially, for serial decimal dilutions in quintuplicate, from J. Bacteriol. 25 101 (1933), page 400, "III. MPN (Most Probable Number) Table", thereby the most probable number of 0.79 is read from $P_1 = 5$, $P_2 = 3$, and $P_3 = 0$. This value is the number of the template DNA derived by a probabilistic statistical technique. By multiplying MPN with the reciprocal of the critical dilution degree and consideration of the initial dilution degree, the number of the template DNA contained in the soil is estimated as $0.79 \times 10^5 \times 10^2 = 0.79 \times 10^7$. This number of the template DNA itself is the number of the microorganism individuals (cell number) in 1 gram of the soil provided that the sequence of the template DNA is characteristic of the target microorganism in the soil and the

sequence exists only one in number in one microbial cell. Naturally in this example, the highest dilution at which all the wells fluoresces reflects the number of the template nucleic acid. Therefrom, a calibration curve for the fluorescence intensity as the function of the number of the template DNA (or number of the individual microorganism) can be made.

Table 1

MPN (Most Probable Number), for Serial decimal Dilution, in quintuplicate							
P_1	P_2	P_3					
		0	1	2	3	4	5
5	0	(0.23)	(0.31)	0.43	0.58	0.76	0.95
5	1	(0.33)	(0.46)	0.64	0.84	1.1	1.3
5	2	(0.49)	(0.70)	(0.95)	1.2	1.5	1.8
5	3	(0.79)	(1.1)	(1.4)	1.8	2.1	2.5
5	4	(1.3)	(1.7)	(2.2)	(2.8)	3.5	4.3
5	5	(2.4)	(3.5)	(5.4)	(9.2)	(16)	-

In Table 1, the numbers in parentheses are of high reliability (probability L is 0.05 or lower). When a code without parentheses is obtained for the experimental results, the process of the experiment needs to be reconfirmed. The MPN table cited as Table 1 is a part of the MPN table of the aforementioned literature. If an experimental result falls outside Table 1, corresponding part of the original MPN table of the literature should be referred to. The literature includes MPN tables for various experimental conditions, which may be used according to the experiment conditions.

In the conventional MPN-PCR method in which PCR and agarose gel electrophoresis are combined as described in Related Background Art, two steps of PCR are conducted: the first PCR operation for determining the dilution limit for the positive reaction, and the second PCR operation to investigate the vicinity of the critical dilution in detail to obtain the most probable number of the individuals from the MPN table. For example, from the above-described soil sample (1 gram), DNA is recovered in the same manner as above. The recovered DNA, without the above-described first dilution, is serially diluted to the dilution degrees of from 10^{-1} to 10^{-3}. The respective dilution solutions are subjected to the first PCR in a conventional manner, and the presence of the amplification product is detected by agarose gel electrophoresis to find that the dilutions are positive to the 10^{-7} dilution degree. Then 5 samples of the respective dilutions at 10^{-6}, 10^{-7}, and 10^{-8} dilution are again subjected to PCR. The positive numbers at each dilution are 5/5 for 10^{-6} dilution, 3/5 for 10^{-7} dilution, and 0/5 for 10^{-8} dilution. Therefore the value 0.79 is obtained from the MPN table in the same manner as above. The number of the template DNA in the soil is derived from this value multiplied by the reciprocal of the dilution degree of the dilution limit (0.79×10^7). This method, however, requires two series of operation of PCR and electrophoresis, and is troublesome. On the other hand, the method of the present invention gives the results directly by conducting one PCR operation without the electrophoresis operation, thereby the measurement operation being simplified greatly.

When the amplification product of PCR in the MPN-PCR method is detected with the aforementioned dye compound, the aforementioned operations and conditions can be employed in the determination of the PCR amplification product, as well as the aforementioned kit.

In the MPN-PCR method, use of a tool having a plurality of reaction chambers (wells) such as a microplate is preferred since simultaneous treatment of serially diluted samples is required for statistical treatment of the result. Even when PCR is conducted in different vessels, the microplate is preferably used for the detection of the amplification product with the dye compound.

This method for measuring the number of the template DNA is applicable effectively to measure the cell number of the target microorganism in a liquid or in soil, the number of microbial cells in various samples, the number of cells in various samples, the number of cells in various tissues, the number of a specified gene and copy number thereof in various living body samples, and so forth. Usually, the concentration of template DNA is estimated by weight based on the light absorption intensity. In the case of cancer cells, however, the number of the copies (the number of specified genes) is frequently of interest. In such a case, the method of the present invention is useful. The number of copies of a specified gene in a tissue can be estimated according to the present invention, irrespectively of the amount of the cells from which the nucleic acid is extracted, by measuring the number of a common gene in the same manner as

described above, then measuring the number of a specified gene such as a oncogene, and deriving a ratio of the number of the specified gene and the number of the common gene which is common to normal cells and cancer cells.

This method is also useful for judging the presence of cancer cells in a human tissue. For this purpose, for example, the number of a specified gene common to all the cells constituting the tissue is estimated by this method, and the number of a specific gene characteristic to cancer cells to be detected is estimated for the same tissue sample.

Reference Example 1

100 ml of acetic anhydride and 30 ml of concentrated sulfuric acid were mixed with cooling, and the resulting mixture was kept at 80 °C for 3 hours. Thereto 20 ml of acetic anhydride and 30 ml of p-dimethylaminoacetophenone were added at room temperature. Then the mixture was stirred at 45 °C for 24 hours for reaction. An equal amount of ethanol was added thereto, and the mixture was cooled. Further thereto, aqueous potassium iodide solution was added to precipitate a crude crystalline matter. This crude crystalline matter was collected by filtration, and recrystallized from an ethanol-ether mixture (1:4 in volume ratio) to obtain 2-methyl-4,6-bis-(4-N,N-dimethylaminophenyl)pyrylium iodide (Compound 1 in Table 2, where Y is I) in a green crystal form.

[Analysis results of obtained Compound 1 (Y:I)]

Melting point: 254 - 257 °C
UV/visible (CH_3CN, $\epsilon \times 10^{-4}$) λmax: 444 nm (2.43), 550 nm (8.24)
NMR (^1H, DMSO) δppm: 8.3737 (1H, s), 8.2729 (1H, d, J = 9.0 Hz), 8.1795 (1H, d, J = 9.0 Hz), 7.8864(1H, s), 6.9117 (4H, t, $J_{AB} = J_{BC} = 9.77$), 3.1829 (6H, s), 3.1340 (6H,s), 2.6809 (3H, s)
FAB mass m/z 333
IR (KBr) ν cm^{-1}: 1645, 1610(sh), 1580(s), 1490(s), 1270, 1200, 1160

2-methyl-4,6-bis-(4-N,N,-dimethylaminophenyl)pyrylium perchlorate (Compound 1 (Y: ClO_4)) was prepared in the same manner as above except that the aqueous potassium iodide was replaced by aqueous perchlorate solution.

Reference Example 2

20 Grams of sodium sulfide nonahydrate was dissolved in deionized water, and the total volume was adjusted to 50 ml. In this solution, 7 g of sodium hydrogen carbonate was dissolved. Further thereto, 50 ml of ethanol was added under cooling with ice. The mixture was stirred at room temperature for 30 minutes. The precipitated sodium carbonate was removed by filtration, and washed with 25 ml of ethanol. The filtrate and the washing were combined to obtain about 125 ml of a sodium hydrogen sulfide solution in ethanol.

0.92 Gram of 2-methyl-4,6-bis-(4-N,N-dimethylaminophenyl)pyrylium iodide prepared in Reference Example 1 was dissolved in 20 ml of DMSO. To the resulting solution, to which 5 ml of the above sodium hydrogen sulfide solution in water-ethanol was added. The mixture was stirred at room temperature for 5 minutes. Then 0.75 ml of hydroiodic acid was added and the mixture was stirred 5 minutes. Thereafter in a conventional manner, the mixture was extracted with dichloromethane, purified by silica gel column chromatography, and the product was recrystallized from a ethanol-ether mixed solvent (1:4 in volume ratio) to obtain 0.7 g of crystalline 2-methyl-4,6-bis-(4-N,N-dimethylaminophenyl)thiopyrylium iodide (Compound 2 in Table 2, where Y is I).

[Analysis results of obtained Compound 2 (Y:I)]

Melting point: 246 - 248 °C
UV/visible (CH_3CN, $\epsilon \times 10^{-4}$) λmax: 495 nm (2.50), 587 nm (4.95)
NMR (^1H, DMSO) δppm: 8.5679 (1H, s), 8.4323 (1H, s), 8.2436 (2H, d, J = 9.27 Hz), 7.9786 (2H, d, J = 9.28), 6.8959 (4H, t, $J_{AB} = J_{BC} = 9.28$), 3.1756 (6H, s), 3.1157 (6H,s), 2.8323 (3H, s)
FAB mass m/z 349
IR (Kbr) ν cm^{-1}: 1600(s), 1560(s), 1640(s), 1430(s), 1370(s), 1260(s), 1160(s)

2-methyl-4,6-bis-(4-N,N,-dimethylaminophenyl)thiopyrylium perchlorate (Compound 2 (Y: ClO_4)) was prepared in the same manner as above except that the aqueous potassium iodide was replaced by an aqueous perchlorate solution.

Reference Example 3

Compounds 3 to 55 shown in Table 2 were prepared respectively. In Table 2, Φ represents a p-phenylene group, or a phenyl group.

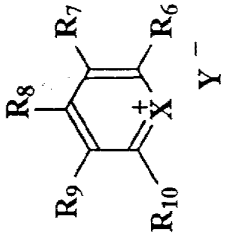


Table 2

Compound No.	X	Y	R_i	L	A
1	O	ClO_4 or I	$R_6 = CH_3$ $R_7 = H$ $R_8 = \Phi - N(CH_3)_2$ $R_9 = H$ $R_{10} = A$		$\Phi - N(CH_3)_2$
2	S	ClO_4 or I	$R_6 = CH_3$ $R_7 = H$ $R_8 = \Phi - N(CH_3)_2$ $R_9 = H$ $R_{10} = A$		$\Phi - N(CH_3)_2$

Table 2 (continued)

Compound No.	X	Y	R_i	L	A
3	O	ClO_4 or I	$R_6 = \Phi$ $R_7 = H$ $R_8 = A$ $R_9 = H$ $R_{10} = \Phi$		$\Phi-N(CH_3)_2$
4	S	ClO_4 or I	$R_6 = \Phi$ $R_7 = H$ $R_8 = A$ $R_9 = H$ $R_{10} = \Phi$		$\Phi-N(CH_3)_2$
5	O	ClO_4 or I	$R_6 = \Phi$ $R_7 = H$ $R_8 = L-A$ $R_9 = H$ $R_{10} = \Phi$	general formula [II] n = 0 Z = H	$\Phi-N(CH_2CH_3)_2$

Table 2 (continued)

Compound No.	X	Y	R_i	L	A
6	S	ClO_4 or I	$R_6 = \Phi$ $R_7 = H$ $R_8 = L-A$ $R_9 = H$ $R_{10} = \Phi$	general formula [II] n = 0 Z = H	$\Phi-N(CH_2CH_3)_2$
7	O	ClO_4 or I	$R_6 = \Phi$ $R_7 = H$ $R_8 = \Phi$ $R_9 = H$ $R_{10} = L-A$	general formula [II] n = 0 Z = H	$\Phi-N(CH_3)_2$
8	S	ClO_4 or I	$R_6 = \Phi$ $R_7 = H$ $R_8 = \Phi$ $R_9 = H$ $R_{10} = L-A$	general formula [II] n = 0 Z = H	$\Phi-N(CH_3)_2$

Table 2 (continued)

Compound No.	X	Y	R_i	L	A
9	O	ClO_4 or I	$R_6 = \Phi$ $R_7 = H$ $R_8 = L-A$ $R_9 = H$ $R_{10} = \Phi$	general formula [II] n = 1 Z = H	$\Phi-N(CH_3)_2$
10	S	ClO_4 or I	$R_6 = \Phi$ $R_7 = H$ $R_8 = L-A$ $R_9 = H$ $R_{10} = \Phi$	general formula [II] n = 1 Z = H	$\Phi-N(CH_3)_2$
11	O	ClO_4 or I	$R_6 = \Phi$ $R_7 = H$ $R_8 = L-A$ $R_9 = H$ $R_{10} = \Phi$	general formula [II] n = 1 $Z = (-)CH=CH-\Phi-N(CH_3)_2$	$\Phi-N(CH_3)_2$

Table 2 (continued)

Compound No.	X	Y	R_i	L	A
12	S	ClO_4 or I	$R_6 = \Phi$ $R_7 = H$ $R_8 = L-A$ $R_9 = H$ $R_{10} = \Phi$	general formula [II] n = 1 $Z = (-)CH=CH-\Phi-N(CH_3)_2$	$\Phi-N(CH_3)_2$
13	O	ClO_4 or I	$R_6 = \Phi$ $R_7 = H$ $R_8 = L-A$ $R_9 = H$ $R_{10} = \Phi$	general formula [III] n = 1	$\Phi-N(CH_3)_2$
14	S	ClO_4 or I	$R_6 = \Phi$ $R_7 = H$ $R_8 = L-A$ $R_9 = H$ $R_{10} = \Phi$	general formula [III] n = 1	$\Phi-N(CH_3)_2$

Table 2 (continued)

Compound No.	X	Y	R_i	L	A
15	O	ClO_4 or I	$R_6 = \Phi$ $R_7 = H$ $R_8 = L-A$ $R_9 = H$ $R_{10} = \Phi$	general formula [IV]	$\Phi-N(CH_2CH_3)_2$
16	S	ClO_4 or I	$R_6 = \Phi$ $R_7 = H$ $R_8 = L-A$ $R_9 = H$ $R_{10} = \Phi$	general formula [IV]	$\Phi-N(CH_2CH_3)_2$
17	O	ClO_4 or I	$R_6 = \Phi$ $R_7 = H$ $R_8 = \Phi$ $R_9 = H$ $R_{10} = L-A$	general formula [IV]	$\Phi-N(CH_2CH_3)_2$

Table 2 (continued)

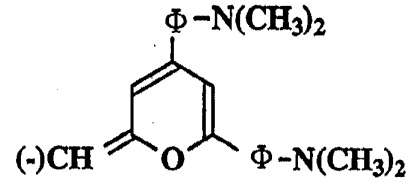
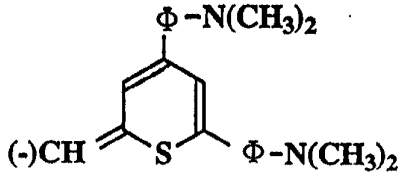
Compound No.	X	Y	R_i	L	A
18	S	ClO_4 or I	$R_6 = \Phi$ $R_7 = H$ $R_8 = \Phi$ $R_9 = H$ $R_{10} = L-A$	general formula [IV]	$\Phi-N(CH_2CH_3)_2$
19	O	ClO_4 or I	$R_6 = \Phi$ $R_7 = H$ $R_8 = \Phi$ $R_9 = H$ $R_{10} = L-A$	general formula [V]	
20	S	ClO_4 or I	$R_6 = \Phi$ $R_7 = H$ $R_8 = \Phi$ $R_9 = H$ $R_{10} = L-A$	general formula [V]	

Table 2 (continued)

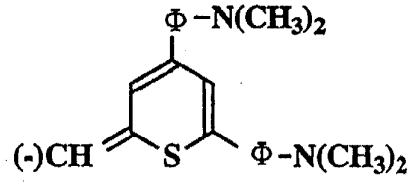
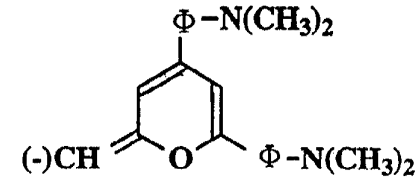
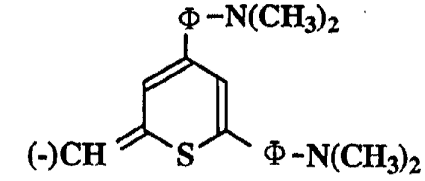
Compound No.	X	Y	R_i	L	A
21	O	ClO_4 or I	$R_6 = \Phi$ $R_7 = H$ $R_8 = \Phi$ $R_9 = H$ $R_{10} = L-A$	general formula [V]	
22	O	ClO_4 or I	$R_6 = \Phi$ $R_7 = H$ $R_8 = \Phi$ $R_9 = H$ $R_{10} = L-A$	general formula [VI]	
23	S	ClO_4 or I	$R_6 = \Phi$ $R_7 = H$ $R_8 = \Phi$ $R_9 = H$ $R_{10} = L-A$	general formula [VI]	

Table 2 (continued)

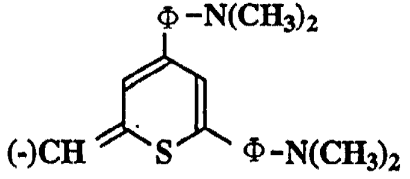
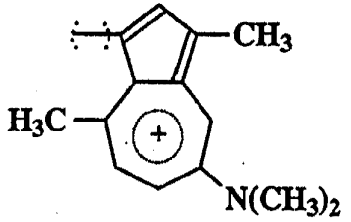
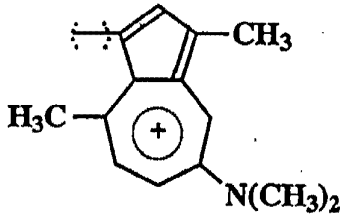
Compound No.	X	Y	R_i	L	A
24	O	ClO_4 or I	$R_6 = \Phi$ $R_7 = H$ $R_8 = \Phi$ $R_9 = H$ $R_{10} = L-A$	general formula [VI]	
25	O	ClO_4 or I	$R_6 = \Phi$ $R_7 = H$ $R_8 = \Phi$ $R_9 = H$ $R_{10} = L-A$	general formula [II] n = 0 Z = H	
26	S	ClO_4 or I	$R_6 = \Phi$ $R_7 = H$ $R_8 = \Phi$ $R_9 = H$ $R_{10} = L-A$	general formula [II] n = 0 Z = H	

Table 2 (continued)

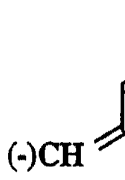
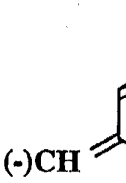
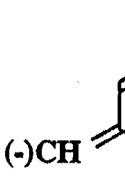
Compound No.	X	Y	R_i	L	A
27	O	ClO_4 or I	$R_6 = \Phi$ $R_7 = H$ $R_8 = \Phi$ $R_9 = H$ $R_{10} = L-A$	general formula [II] n = 0 Z = H	
28	S	ClO_4 or I	$R_6 = \Phi$ $R_7 = H$ $R_8 = \Phi$ $R_9 = H$ $R_{10} = L-A$	general formula [II] n = 0 Z = H	
29	O	ClO_4 or I	$R_6 = \Phi$ $R_7 = H$ $R_8 = \Phi$ $R_9 = H$ $R_{10} = L-A$	general formula [II] n = 0 Z = H	

Table 2 (continued)

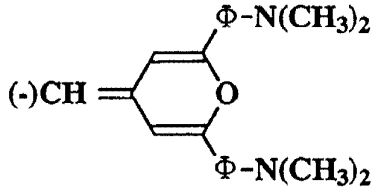
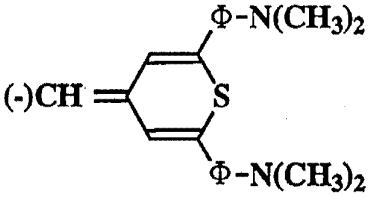
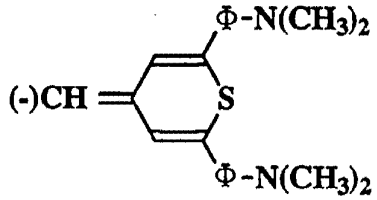
Compound No.	X	Y	R_i	L	A
30	O	ClO_4 or I	$R_6 = \Phi$ $R_7 = H$ $R_8 = L-A$ $R_9 = H$ $R_{10} = \Phi$	general formula [II] n = 0 Z = H	
31	S	ClO_4 or I	$R_6 = \Phi$ $R_7 = H$ $R_8 = L-A$ $R_9 = H$ $R_{10} = \Phi$	general formula [II] n = 0 Z = H	
32	O	ClO_4 or I	$R_6 = \Phi$ $R_7 = H$ $R_8 = L-A$ $R_9 = H$ $R_{10} = \Phi$	general formula [II] n = 0 Z = H	

Table 2 (continued)

Compound No.	X	Y	R_i	L	A
33	O or S	ClO_4 or I	$R_6 = \Phi-N(CH_3)_2$ $R_7 = H$ $R_8 = A$ $R_9 = H$ $R_{10} = \Phi-N(CH_3)_2$		$\Phi-N(CH_3)_2$
34	O or S	ClO_4 or I	$R_6 = \Phi-N(CH_3)_2$ $R_7 = H$ $R_8 = A$ $R_9 = H$ $R_{10} = \Phi-N(CH_3)_2$		CH_3
35	O or S	ClO_4 or I	$R_6 = \Phi-N(CH_3)_2$ $R_7 = H$ $R_8 = A$ $R_9 = H$ $R_{10} = \Phi-N(CH_3)_2$		$\Phi-COOH$

Table 2 (continued)

Compound No.	X	Y	R_i	L	A
36	O or S	ClO_4 or I	$R_6 = \Phi-N(CH_3)_2$ $R_7 = H$ $R_8 = L-A$ $R_9 = H$ $R_{10} = \Phi-N(CH_3)_2$	general formula [II] n = 0 Z = H	$\Phi-N(CH_3)_2$
37	O or S	ClO_4 or I	$R_6 = \Phi-N(CH_3)_2$ $R_7 = H$ $R_8 = L-A$ $R_9 = H$ $R_{10} = \Phi-N(CH_3)_2$	general formula [II] n = 1 Z = H	$\Phi-N(CH_3)_2$
38	O or S	ClO_4 or I	$R_6 = \Phi-N(CH_3)_2$ $R_7 = H$ $R_8 = L-A$ $R_9 = H$ $R_{10} = \Phi-N(CH_3)_2$	general formula [III] n = 1	$\Phi-N(CH_3)_2$

Table 2 (continued)

Compound No.	X	Y	R_i	L	A
39	O or S	ClO_4 or I	$R_6 = \Phi-N(CH_3)_2$ $R_7 = H$ $R_8 = L-A$ $R_9 = H$ $R_{10} = \Phi-N(CH_3)_2$	general formula [IV]	$\Phi-N(CH_3)_2$
40	O or S	ClO_4 or I	$R_6 = \Phi-N(CH_3)_2$ $R_7 = H$ $R_8 = L-A$ $R_9 = H$ $R_{10} = \Phi-N(CH_3)_2$	general formula [II] n = 0 Z = H	$\Phi-COOH$
41	O or S	ClO_4 or I	$R_6 = \Phi-N(CH_3)_2$ $R_7 = H$ $R_8 = L-A$ $R_9 = H$ $R_{10} = \Phi-N(CH_3)_2$	general formula [II] n = 1 Z = H	$\Phi-COOH$

Table 2 (continued)

Compound No.	X	Y	R_i	L	A
42	O or S	ClO_4 or I	$R_6 = \Phi-N(CH_3)_2$ $R_7 = H$ $R_8 = L-A$ $R_9 = H$ $R_{10} = \Phi-N(CH_3)_2$	general formula [III] n = 1	$\Phi-COOH$
43	O or S	ClO_4 or I	$R_6 = \Phi-N(CH_3)_2$ $R_7 = H$ $R_8 = L-A$ $R_9 = H$ $R_{10} = \Phi-N(CH_3)_2$	general formula [IV]	$\Phi-COOH$
44	O or S	ClO_4 or I	$R_6 = L-A$ $R_7 = H$ $R_8 = \Phi-N(CH_3)_2$ $R_9 = H$ $R_{10} = \Phi-N(CH_3)_2$	general formula [II] n = 0 Z = H	$\Phi-N(CH_3)_2$

Table 2 (continued)

Compound No.	X	Y	R_i	L	A
45	O or S	ClO_4 or I	$R_6 = L-A$ $R_7 = H$ $R_8 = \Phi-N(CH_3)_2$ $R_9 = H$ $R_{10} = \Phi-N(CH_3)_2$	general formula [II] n = 1 Z = H	$\Phi-N(CH_3)_2$
46	O or S	ClO_4 or I	$R_6 = L-A$ $R_7 = H$ $R_8 = \Phi-N(CH_3)_2$ $R_9 = H$ $R_{10} = \Phi-N(CH_3)_2$	general formula [III] n = 1	$\Phi-N(CH_3)_2$
47	O or S	ClO_4 or I	$R_6 = L-A$ $R_7 = H$ $R_8 = \Phi-N(CH_3)_2$ $R_9 = H$ $R_{10} = \Phi-N(CH_3)_2$	general formula [IV]	$\Phi-N(CH_3)_2$

Table 2 (continued)

Compound No.	X	Y	R_i	L	A
48	O or S	ClO_4 or I	$R_6 = L-A$ $R_7 = H$ $R_8 = \Phi-N(CH_3)_2$ $R_9 = H$ $R_{10} = \Phi-N(CH_3)_2$	general formula [II] n = 0 Z = H	$\Phi-COOH$
49	O or S	ClO_4 or I	$R_6 = L-A$ $R_7 = H$ $R_8 = \Phi-N(CH_3)_2$ $R_9 = H$ $R_{10} = \Phi-N(CH_3)_2$	general formula [II] n = 1 Z = H	$\Phi-COOH$
50	O or S	ClO_4 or I	$R_6 = L-A$ $R_7 = H$ $R_8 = \Phi-N(CH_3)_2$ $R_9 = H$ $R_{10} = \Phi-N(CH_3)_2$	general formula [III] n = 1	$\Phi-COOH$

Table 2 (continued)

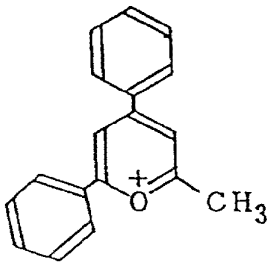
Compound No.	X	Y	R_i	L	A
51	O or S	ClO_4 or I	$R_6 = L-A$ $R_7 = H$ $R_8 = \Phi-N(CH_3)_2$ $R_9 = H$ $R_{10} = \Phi-N(CH_3)_2$	general formula [IV]	$\Phi-COOH$
52	O or S	ClO_4 or I	$R_6 = A$ $R_7 = H$ $R_8 = \Phi-N(CH_3)_2$ $R_9 = H$ $R_{10} = \Phi-N(CH_3)_2$		$-COOH$
53	O or S	ClO_4 or I	$R_6 = A$ $R_7 = H$ $R_8 = \Phi-N(CH_3)_2$ $R_9 = H$ $R_{10} = \Phi-N(CH_3)_2$		$\Phi-COOH$

Table 2 (continued)

Compound No.	X	Y	R_i	L	A
54	O or S	ClO_4 or I	$R_6 = \Phi$ $R_7 = H$ $R_8 = \Phi - N(CH_3)_2$ $R_9 = H$ $R_{10} = \Phi - N(CH_3)_2$		
55	O or S	ClO_4 or I	$R_6 = \Phi - N(CH_3)_2$ $R_7 = H$ $R_8 = \Phi$ $R_9 = H$ $R_{10} = \Phi - N(CH_3)_2$		

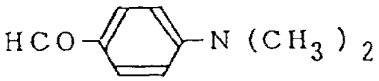
The compounds above were synthesized according known processes as below. The specific reaction operations were conducted in conventional manner.

Compound 7 was prepared by synthesizing Compound [i] according to the method described by W. Foerst et al. ("New Methods of Preparative Organic Chemistry", Acad. Press (1964)),



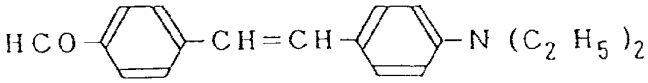
[i]

reacting it with p-N,N-dimethylaminobenzaldehyde (formula shown below):



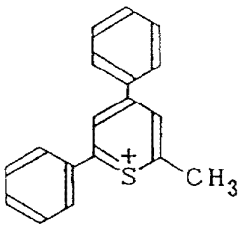
and by reacting the resulting compound with a desired anion.

Compound 17 was prepared by reacting Compound [i] with p-diethylaminostyrylbenzaldehyde (formula shown below):



and reaction the resulting product with a desired anion.

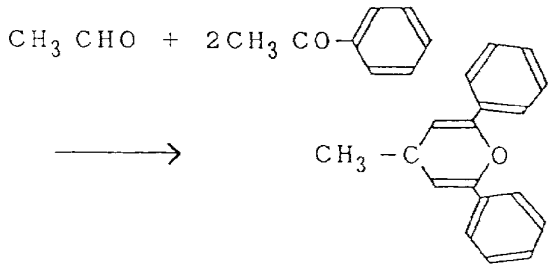
Compound [ii] was prepared by reacting Compound [i] with sodium hydrogen sulfide.

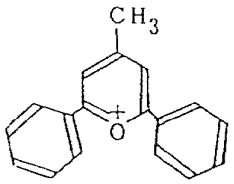


[ii]

From this Compound [ii], Compounds 8 and 18 were prepared in the same manner as Compounds 7 and 17.

Compound [iii] was synthesized from acetophenone and acetaldehyde according to the method described by R. Wizinger (Helv. Chim. Acta, 39 217 (1956)) through the route shown below:





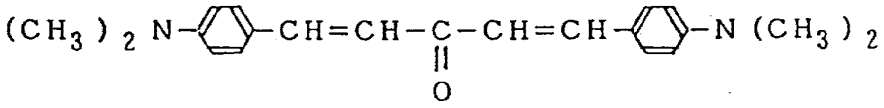
[iii]

Compound 5 was prepared by reacting Compound [iii] with p-dimethylaminobenzaldehyde, and further reacting the resulting compound with a desired anion.

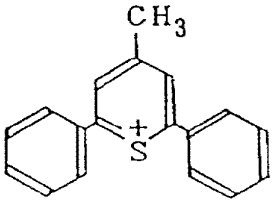
Compound 15 was prepared in the same manner by using p-diethylaminostyrylbenzaldehyde.

Compound 9 was prepared in the same manner by using p-dimethylaminocinnamaldehyde.

Compound 11 was prepared in the same manner by using the compound below:



Compound [iv] was obtained by reacting Compound [iii] with sodium hydrogen sulfide:

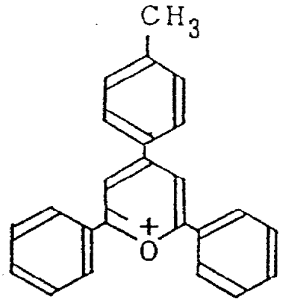


[iv]

Compounds 6, 16, 10, and 12 were prepared respectively in the same manner as Compounds 5, 15, 9, and 11 except that Compound [iv] was used in place of Compound [iii].

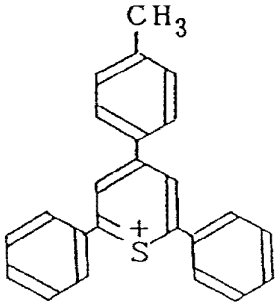
The cation portion of Compound 3 was synthesized in the same manner as Compound [iii] except that p-dimethylaminobenzaldehyde was used in place of acetaldehyde as a starting material. Compound 4 was prepared by reacting the above resulting compound with sodium hydrogen sulfide, and further with a desired anion.

Compound [v] was prepared from p-methylbenzaldehyde and acetophenone in the same manner:



[v]

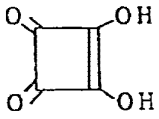
Compound [vi] was prepared by reacting Compound [v] with sodium hydrogen sulfide:



[vi]

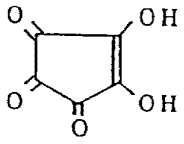
Compounds 13 and 14 were prepared respectively by reacting Compound [v] and Compound [vi] respectively with p-dimethylaminobenzaldehyde, and the resulting compound with a desired anion.

Compounds 19, 20, and 21 were prepared respectively by reacting Compound [i] or [ii] with the cation portion of Compound 1 or 2, and the compound of the formula below:



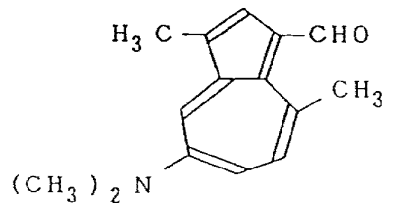
and reacting the product further with a desired anion.

Compounds 22, 23, and 24 were prepared respectively by reacting Compound [i] or [ii] with the cation portion of Compound 1 or 2, and the compound of the formula below:



and reacting the product further with a desired anion.

Compounds 25 and 26 were prepared respectively by reacting Compound [i] or [ii] with the compound of the formula below:



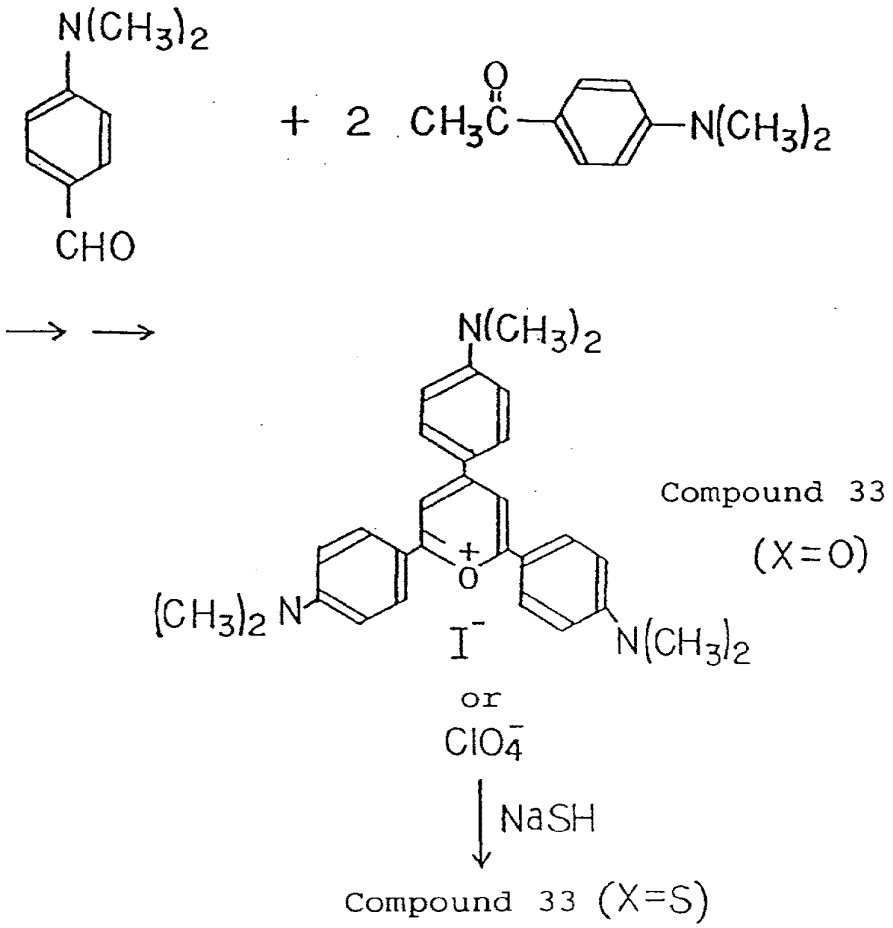
and reacting the product further with a desired anion.

Compounds 27, 28, and 29 were prepared respectively by reacting Compound [i] or [ii] with the cation portion of Compound 1 or 2, and ethyl orthoformate [$HC(OC_2H_5)_3$], and reacting the product further with a desired anion.

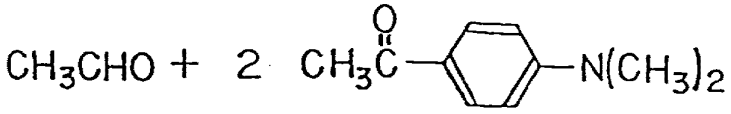
Compounds 30, 31, and 32 were prepared respectively by reacting Compound [iii] or [iv] with a p-dimethylamino derivative of Compound [iii] or [iv] derived in the same manner as Compound [iii] or [iv], and ethyl orthoformate, and reacting the product further with a desired anion.

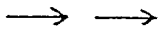
Compounds 33 to 55 were synthesized through the processes below.

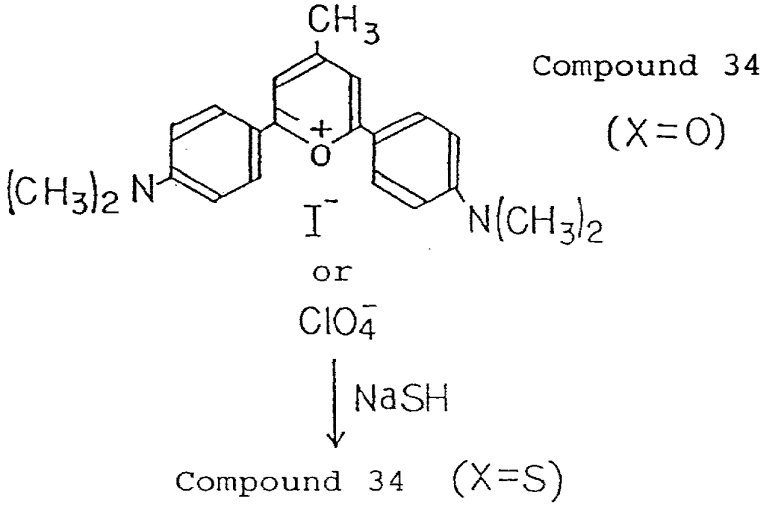
Synthesis of Compound 33



Synthesis of Compound 34



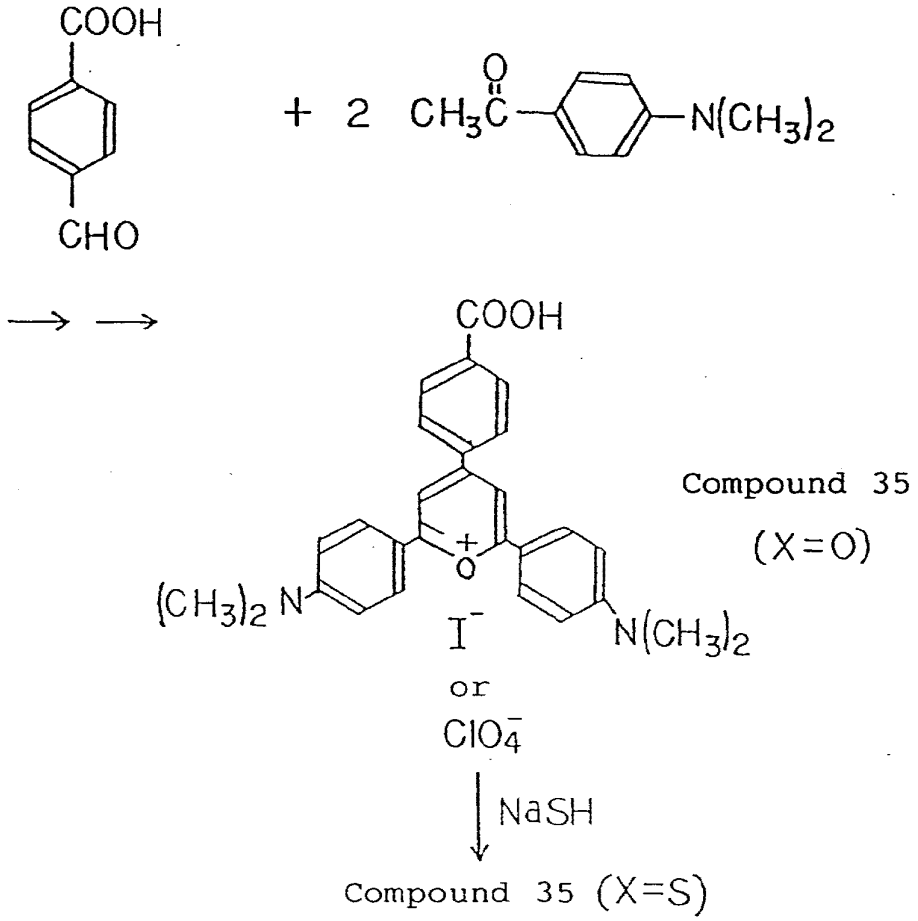




Compound 34 (X=O)

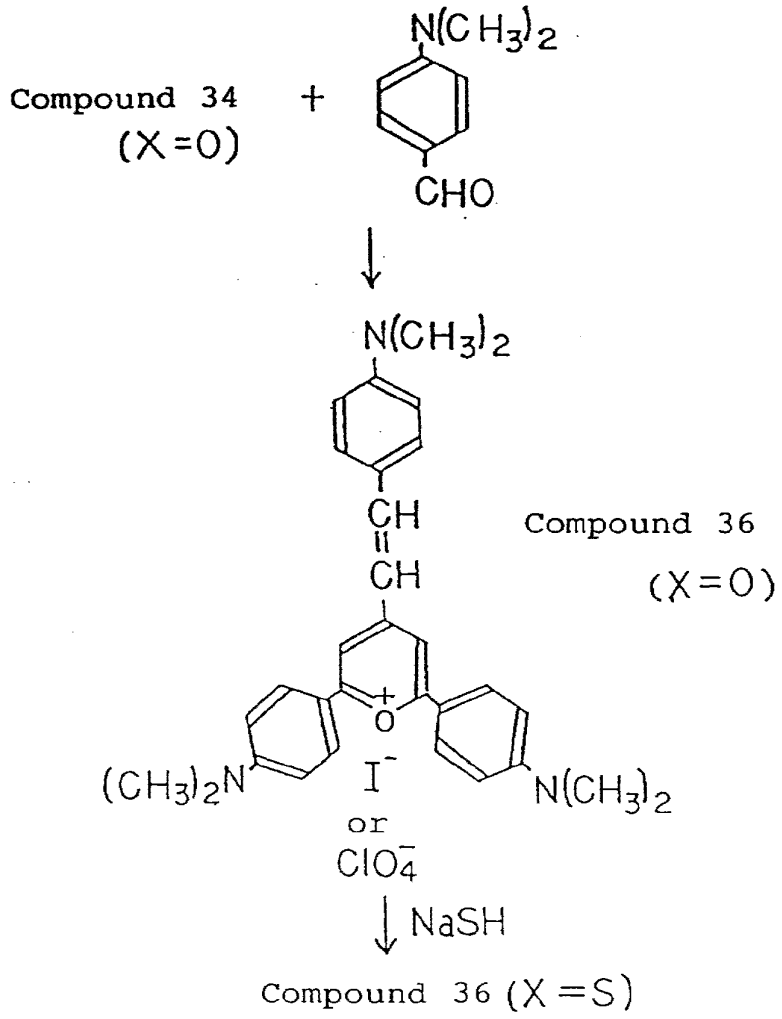
Compound 34 (X=S)

Synthesis of Compound 35

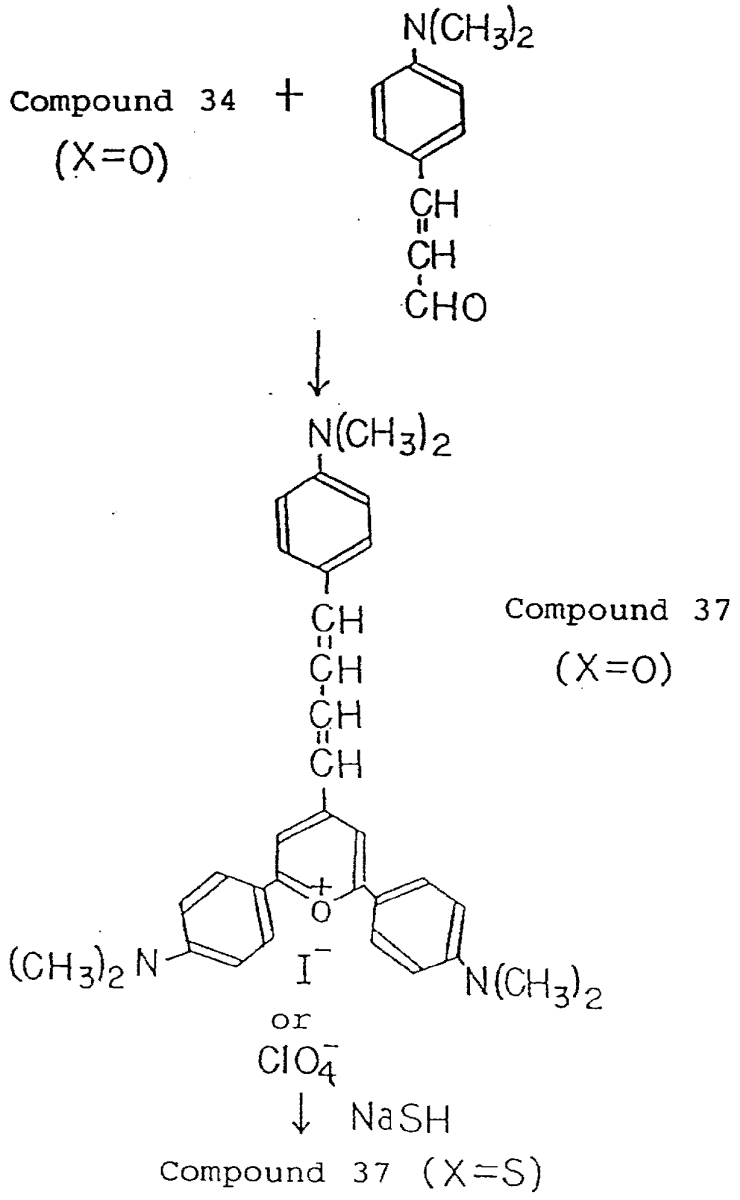


Compound 35 (X=O)

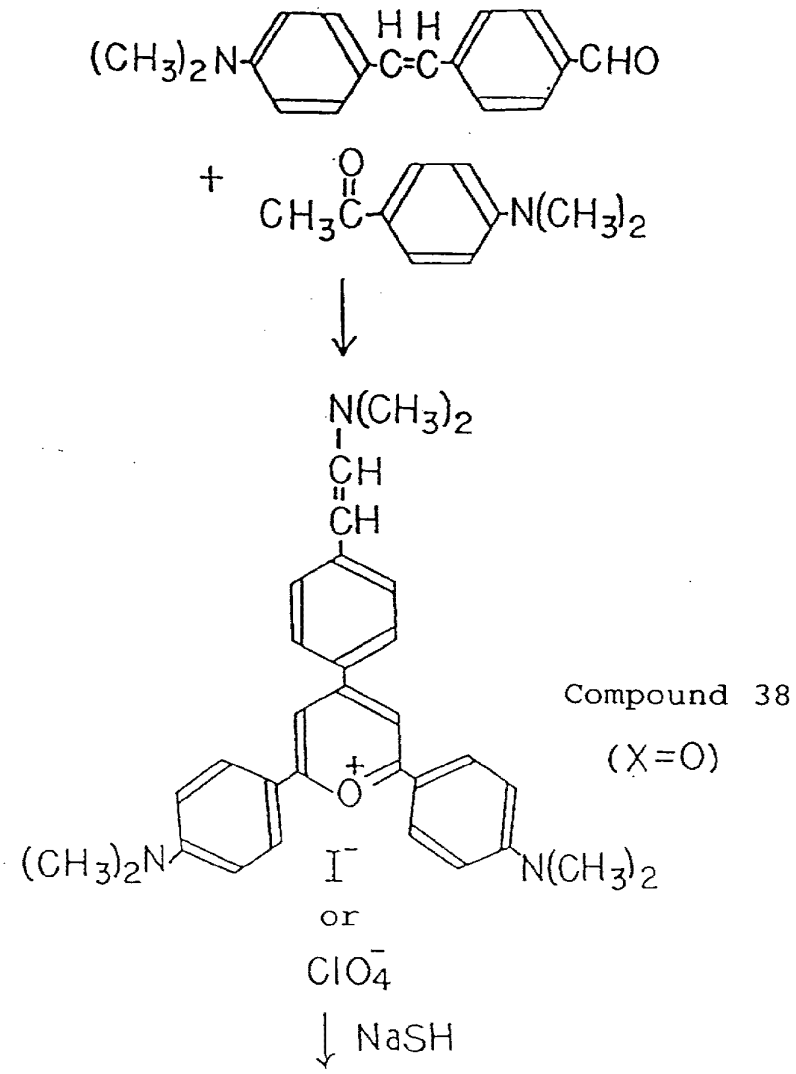
Synthesis of Compound 36



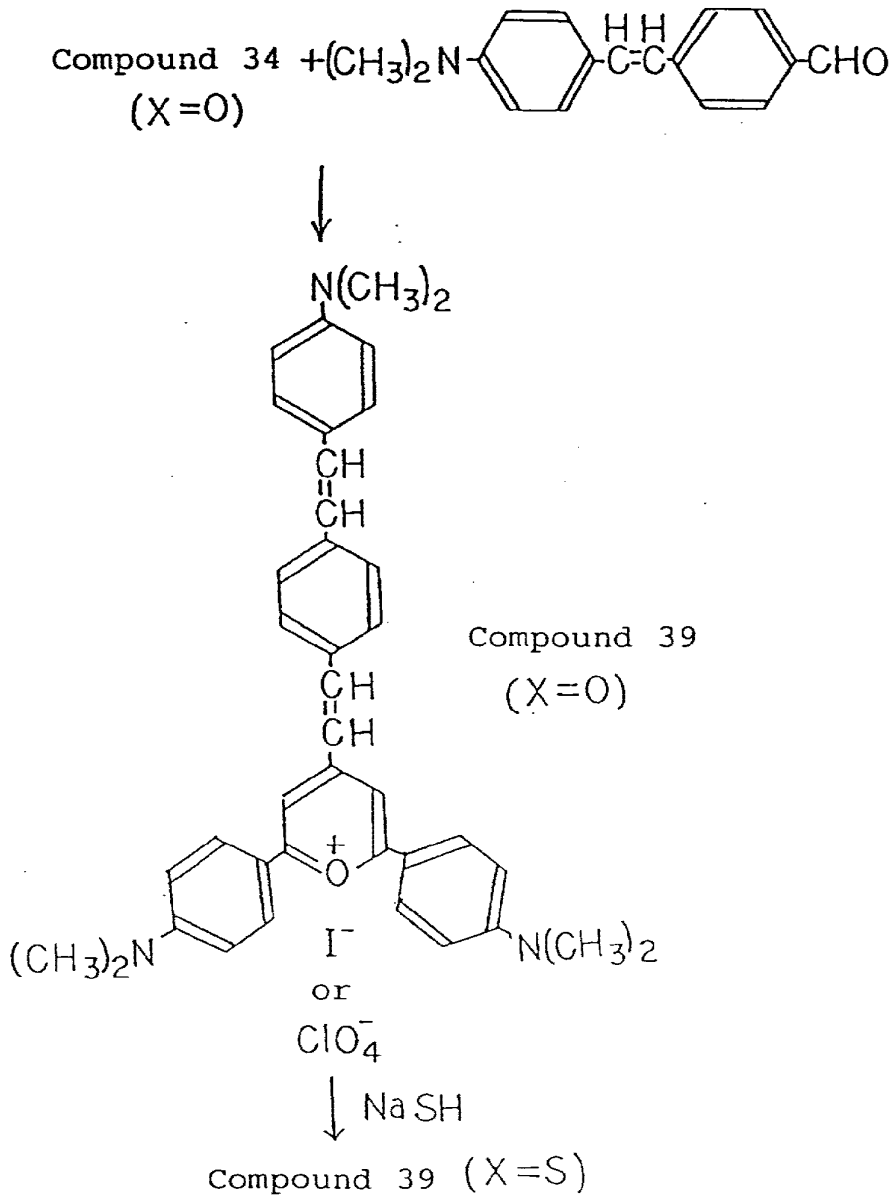
Synthesis of Compound 37



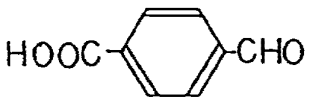
Synthesis of Compound 38



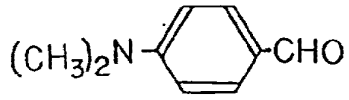
Synthesis of Compound 39



Compound 40 was synthesized in the same manner as Compound 36 except for using the compound

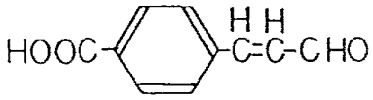


in place of

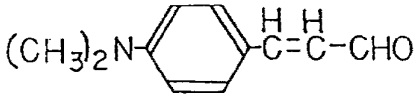


as a starting material.

Compound 41 was synthesized in the same manner as Compound 37 except for using the compound

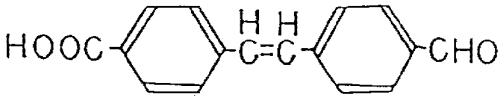


in place of

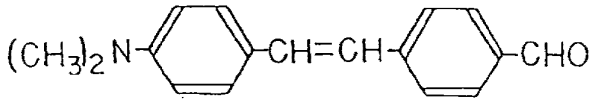


as a starting material.

Compound 42 was synthesized in the same manner as Compound 38 except for using the compound

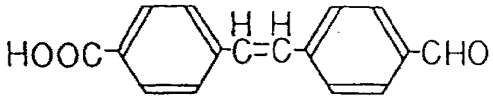


in place of

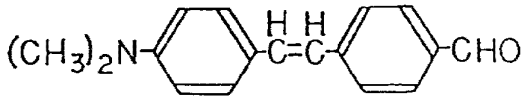


as a starting material.

Compound 43 was synthesized in the same manner as Compound 39 except for using the compound

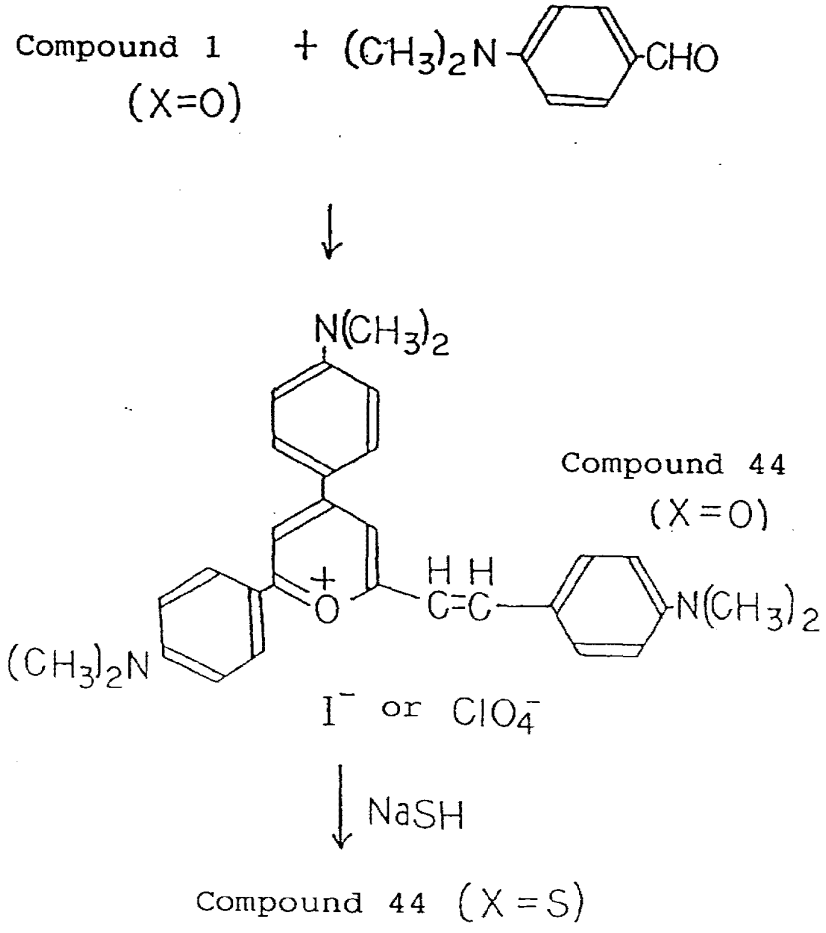


in place of

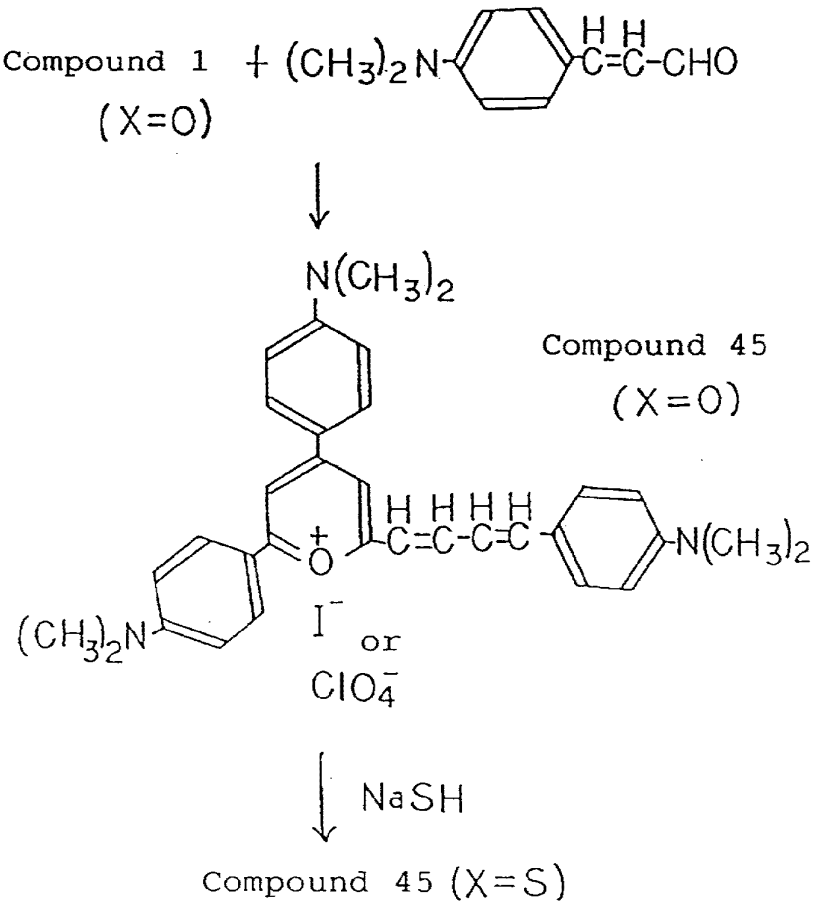


as a starting material.

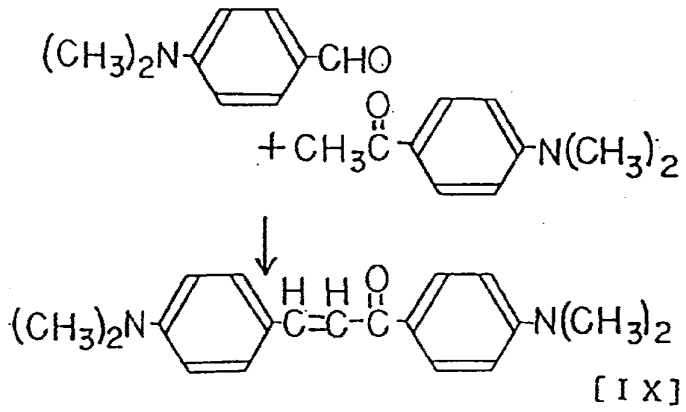
Synthesis of Compound 44



Synthesis of Compound 45

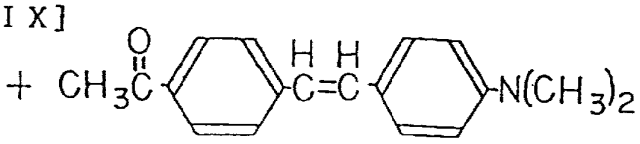


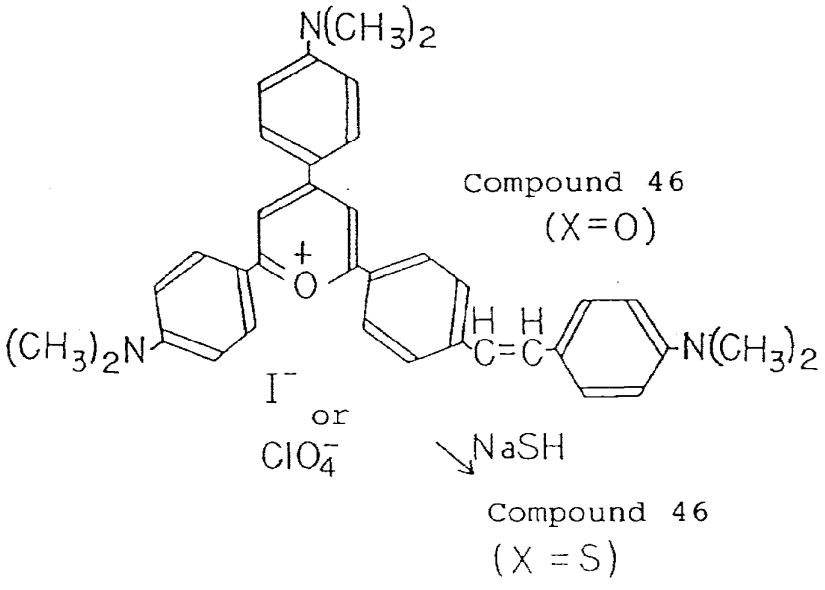
Synthesis of Compound 46



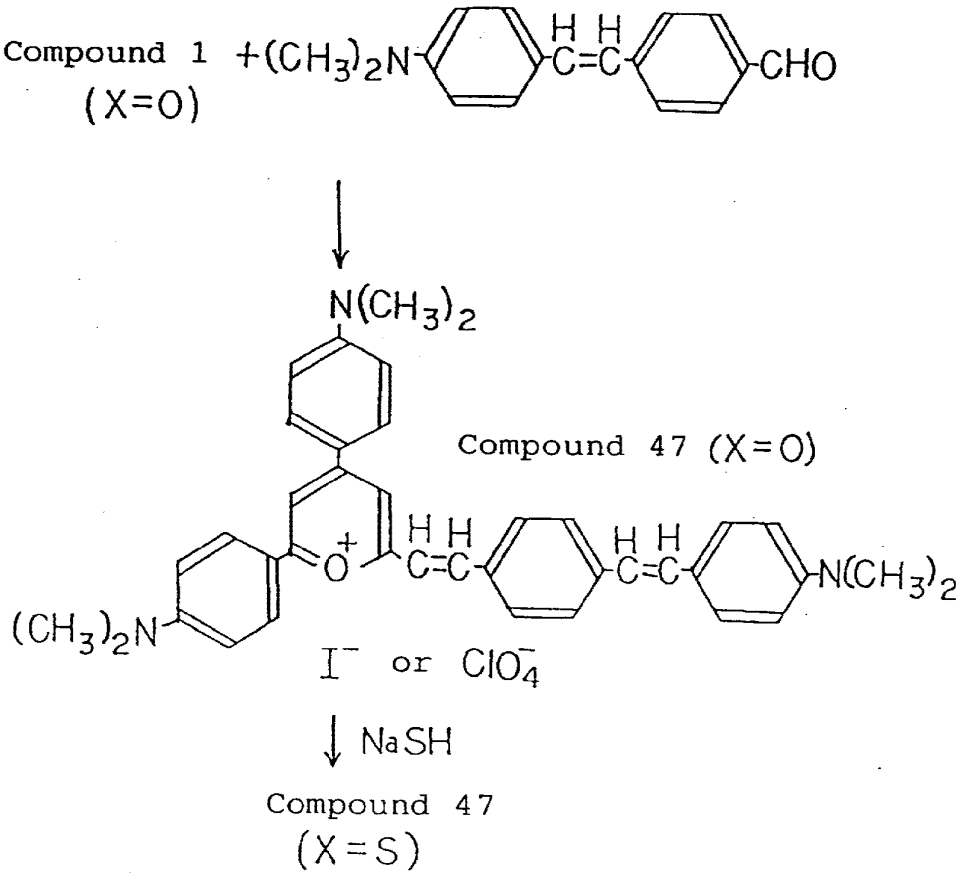
[IX]

Compound [IX]

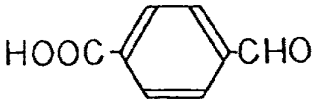




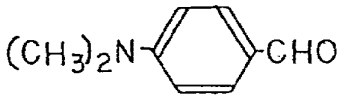
Synthesis of Compound 47



Compound 48 was synthesized in the same manner as Compound 44 except for using the compound

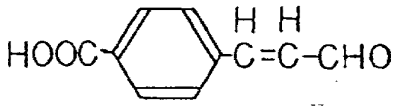


in place of

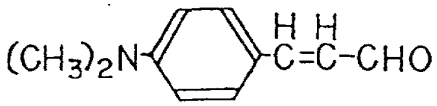


as a starting material.

Compound 49 was synthesized in the same manner as Compound 45 except for using the compound

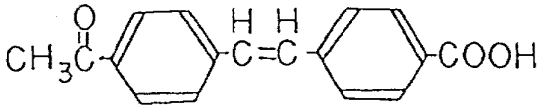


in place of

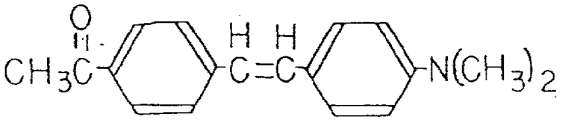


as a starting material.

Compound 50 was synthesized in the same manner as Compound 46 except for using the compound

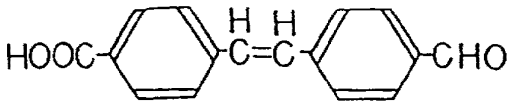


in place of

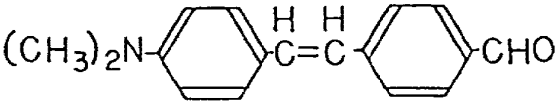


as a starting material.

Compound 51 was synthesized in the same manner as Compound 47 except for using the compound

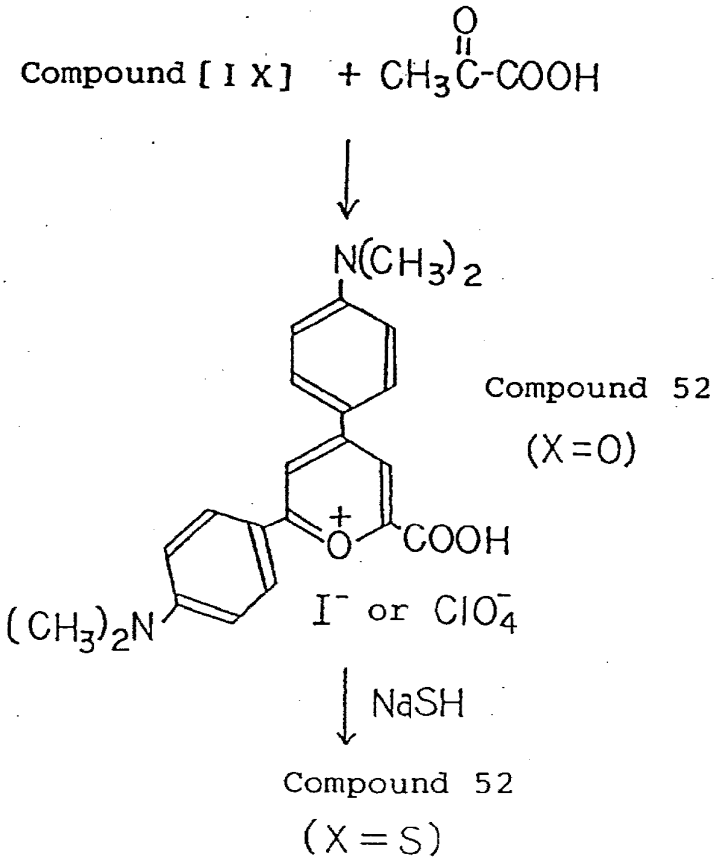


in place of

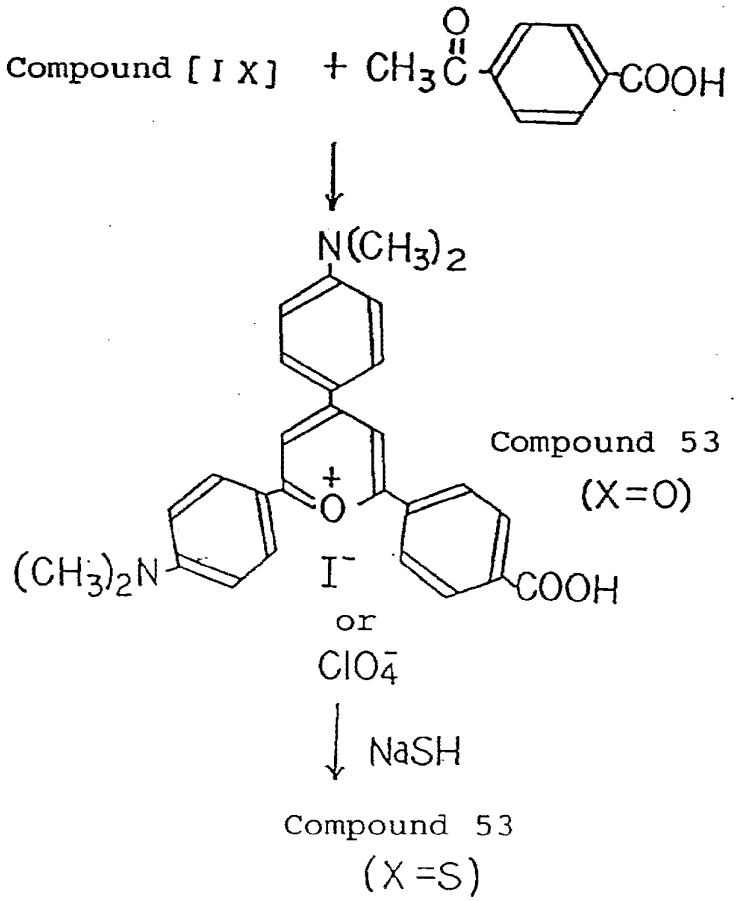


as a starting material.

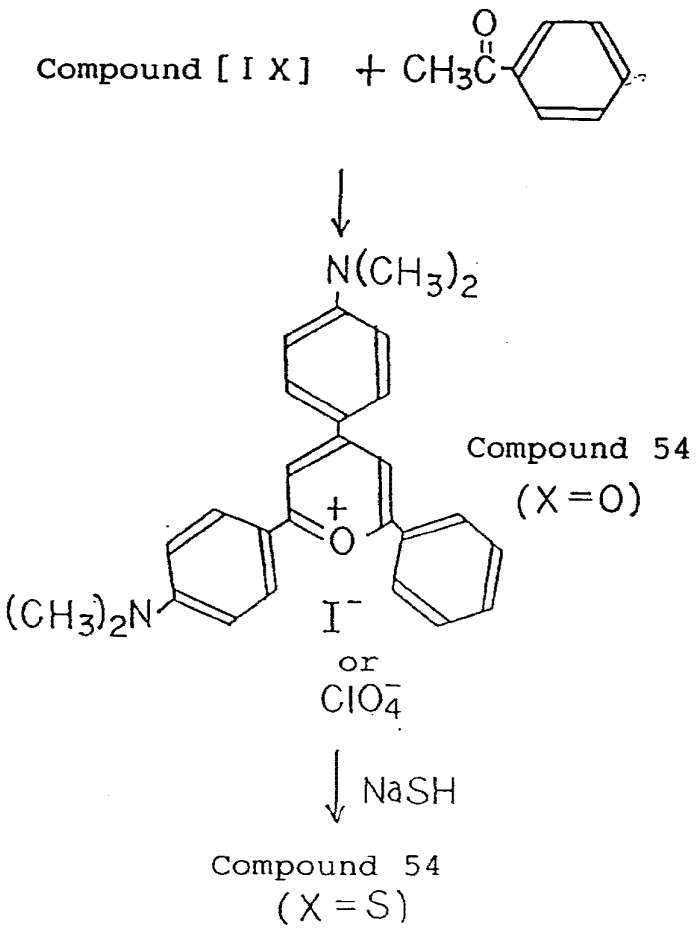
Synthesis of Compound 52



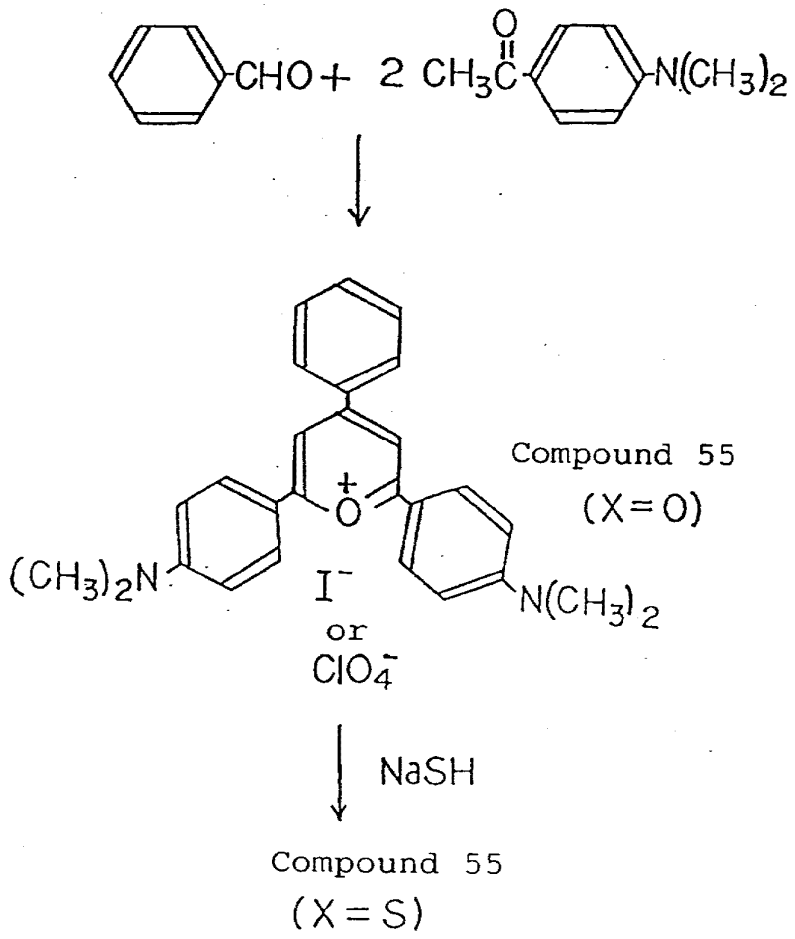
Synthesis of Compound 53



Synthesis of Compound 54



Synthesis of Compound 55



Reference Example 4

Compound 1 obtained in Reference Example 1 was dissolved in acetonitrile to make a stock solution, to which a phosphate buffer was added to the final concentration of 10 mM, followed by dilution with water containing 10% acetonitrile to the final concentration of Compound 1 of 3×10^{-5} M. This solution is referred to as "Sample I". The absorption spectrum of Sample I was measured with a spectrophotometer in a conventional manner.

Salmon sperm DNA (made by Sigma Co.) was dissolved in a TE buffer solution (10 mM Tris - 1mM EDTA), and was purified by phenol extraction. The purified DNA was further digested with restriction enzyme EcoRI for easy handling. An aliquot of this DNA solution was mixed with the stock solution of Compound I to give a DNA concentration of 50 μg/ml and a Compound 1 concentration of 3×10^{-5} M. This solution containing 10% acetonitrile is referred to as "Sample II". The absorption spectrum of Sample II was measured with a spectrophotometer in a conventional manner. In Sample II, the absorption peak of Compound 1 shifted by 20 to 30 nm to longer wavelength owing to its interaction with DNA, which is typical for an intercalator.

When fluorescence spectrum of Sample I and Sample II were taken in a conventional manner, Sample I showed a trace fluorescence peak at about 650 nm by excitation at 550 nm, whereas Sample II containing DNA showed a strong fluorescence peak at about 650 nm with an intensity of about 100 times that of

Sample I at the same excitation wavelength. This shows that Compound 1 is a powerful intercalator.

Solutions were prepared which contain Compound 1 at a concentration of 5×10^{-6} M and DNA at various concentrations by using the above-prepared DNA solution and the above-prepared 10mM phosphate buffer solution containing compound 1 and 10% acetonitrile. The fluorescence intensities of the solutions were measured in a conventional manner to determine the relation to the DNA concentration. The fluorescence intensity increased in proportion to the DNA concentration, the maximum intensity being about 400 times the value in the absence of DNA. The excitation light was emitted from a xenon lamp through a low-cut filter of 480 nm to eliminate ultraviolet light. Further, the compounds shown in Table 2 were subjected to fluorescence intensity measurement in the same manner as above. Typical examples are shown in Table 3.

Table 3

Compound No.	Maximum absorption wavelength (λ_{max})		Fluorescence intensity	
	Absence of DNA	Presence of DNA	λ_{em}	Increase
1	540 nm	560 nm	650 nm	100-fold
2	580 nm	620 nm	700 nm	60-fold
3	535 nm	570 nm	640 nm	13.6-fold
4	575 nm	610 nm	705 nm	10-fold
6	660 nm	690 nm	800 nm	7-fold
8	650 nm	670 nm	weak	-
9	660 nm	720 nm	750 nm	16-fold
11	625 nm	660 nm	735 nm	10-fold
15	670 nm	680 nm	820 nm	5-fold
16	690 nm	720 nm	825 nm	5-fold
17	690 nm	720 nm	weak	-

Example 1 [Determination of nucleic acid with 2,4-bis(4-N,N-dimethylaminophenyl)-6-methylpyrylium salt]

PCR was carried out on 16S ribosomal RNA gene (hereinafter referred to as 16S rRNA gene) of Pseudomonas aeruginosa as the target, and the amplification product was detected by use of 2,4-bis(4-N,N-dimethylaminophenyl)-6-methylpyrylium salt.

Firstly, the entire DNA of P. aeruginosa was prepared as follows. After overnight incubation in a 2×YT culture medium, the cells of 2 ml culture were collected by centrifugation. The collected cells were suspended in 0.5 ml of 0.1M phosphate buffer solution (pH: 8.0), to which 0.05 ml of 10% SDS solution was added. The suspension was mixed well, and was kept standing at 70 °C for one hour. Then the suspension was vortexed to cause complete cell lysis. To this lysate, an equal amount of phenol-chloroform was added, and mixed. Then the mixture was centrifuged, and the upper layer was collected. Thereto ethanol of twice volume was added to recover DNA as precipitate. The DNA was dissolved in 100 µl of TE buffer (pH: 8.0). This DNA was used as the template DNA.

Two primers were used for PCR.

Primer 1: 5' AGAGTTTGATCATGGCTCAG 3' (sequence No. 1)

Primer 2: 5' AACCCAACATCTCACGACAC 3' (sequence No. 2)

These primers were synthesized by means of DNA Synthesizer 381A (made by ABI Co.). The reagents and the techniques for the synthesis are based on the protocol of ABI Co.

PCR was conducted with the template DNA and the primers under the following conditions:

[PCR conditions]

Composition of reaction solution (total volume: 50 μl):	
10× buffer*:	5 μl
dNTPs:	5 μl
Primers 1 and 2:	10 pmol respectively
aaq DNA polymerase:	0.5 unit
Template DNA:	500 pg, 100 pg, 10 pg, 1 pg, 100 fg, or 10 fg

*(100 mM tris-HCl(pH 9.0), 500 mM KCl, 1% Triton X-100, 25 mM $MgCL_2$)

The components were mixed and thereto sterilized water was added to the total volume of 50 μl. The mixture was allowed to react in a Thin-Walled Gene Amp tube (entire volume: 0.5 ml, made by Takara Shuzo Co., Ltd.). Separately, as the blank, a sample was prepared in the same manner as above except that the template DNA was not added. The required amount of the 10× buffer (100 mM tris-HCl(pH 9.0), 500 mM KCl, 1% Triton X-100, 25 mM $MgCL_2$) and the dNTPs were supplied with the polymerase.

Reaction cycle:

Pre-incubation at 92 °C for 5 minutes,
30 cycles of 92 °C for 45 seconds/ 55 °C for 60 seconds/ and 72 °C for 90 seconds,
Final incubation at 72 °C for 5 minutes followed by slow cooling down to 5°C for annealing.

The PCR apparatus employed was Gene PCR System 9600 made by Perkin-Elmer Inc. The amplification product was detected as follows: After the reaction, respective samples are diluted 2-fold, 10-fold, and 20-fold with TE buffer (10 mM Tris-HCl (pH: 8.0) - 1 mM EDTA), to the final volume of 50 μl on a 96-well microtiter plate (Falcon Assay Plate 3911 (U-bottomed well) made by Becton-Dickinson Co.) (See Fig. 1). To each of the wells, was added 1 μl of 150 μg/ml 2,4-bis(4-N,N-dimethylaminophenyl)-6-methylpyrylium iodide solution in acetonitrile. The contents of the wells were mixed well by pipetting. The microplate was set on a Millipore Fluorescence Apparatus (CytoFlour 2350), and using the excitation filter to pass the light of wavelength 590 nm and the emission filter to detect the light of wavelength 645 nm, the fluorescence under the excitation light irradiation was measured. Fig. 2 shows the fluorescence intensity at 645 nm of the respective dilutions as a function of the amount of the template DNA in the reaction. No fluorescence was observed with the blank sample. As shown in Fig. 2, the fluorescence intensity increases as the amount of the template DNA increases, showing the possibility of quantitative determination of template DNA with fluorescence intensity. At the template DNA levels of 100 fg and 10 fg, no PCR amplification product was detected.

The same operation was conducted, but instead of detection with 2,4-bis(4-N,N-dimethylaminophenyl)-6-methylpyrylium salt to the reaction solution in each of the well, a 5 μl aliquot of the respective reaction solutions were taken out and subjected to agarose gel electrophoresis. The gel was stained with ethidium bromide (hereinafter referred to as EB), and the amount of the PCR amplification product was estimated from the fluorescence density of the band. As a result, a distinct band was observed at the template DNA levels of 500 pg, 100 pg, and 10 pg; a weak band was observed at the level of 1 pg; and no PCR amplification product was observed at the levels of 100 fg and 10 fg of template DNA used. At the level of 500 pg, the fluorescence intensity was saturated, giving intensity nearly equal to that of 100 pg template DNA. On the gel,in addition to the band of the PCR amplification product, a fluorescent zone was observed at a lower molecular weight region, which is assumed to come from higher-ordered product formed between the primers.

Comparative Example 1 [Detection of PCR amplification product with EB]

The same operation was conducted as in Example 1 except that EB was used in place of 2,4-bis(4-N,N-dimethylaminophenyl)-6-methylpyrylium salt with the following operation conditions. To each of the wells, was added 1 μl of 250 μg/ml EB solution, and mixed well by pipetting. The mixture was left standing at room temperature for 5 minutes. The fluorescence intensities were measured by means of Millipore Fluorescence Apparatus with a 485nm-light transmitting excitation filter and a 620nm-light transmitting emission filter. Consequently, red fluorescence was observed at all the wells including control wells.

Moreover, no difference of the fluorescence intensity was observed between the sample wells containing the template DNA and the wells not containing template DNA (control wells). Therefore, the quantitative determination was impossible. This was attributed to the presence of higher-order structured matter formed from primers in the sample and blank wells.

Comparative Example 2 [Detection of PCR amplification product employing YOYO-1]

The same operation was conducted as in Example 1 except that YOYO-1 (made by Molecular Probe Co.) was used in place of 2,4-bis(4-N,N-dimethylaminophenyl)-6-methylpyrylium salt with the following operation conditions. The YOYO-1 solution was prepared by diluting 1 mM stock solution 120-fold, and 1 μl of the diluted YOYO-1 solution was added to each of the wells, and mixed well by pipetting. The mixture was left standing at room temperature for 5 minutes. The fluorescence intensities were measured by means of Millipore Fluorescence Apparatus with a 485nm-light transmitting excitation filter and a 530 nm-light transmitting emission filter. Fig. 3 shows the results. As shown in Fig. 3, the fluorescence intensity increased in proportion to the amount of the template DNA, but the increase rate is low. Moreover, the blank wells also produced fluorescence at approximately the same intensity level as those containing the template DNA. The fluorescence of the blank is probably due to the higher-order structured product formed from the primers. Accordingly, in the quantitative determination with this dye, quantitation is not satisfactory because of the small difference in the fluorescence intensity between the reaction mixture and the blank mixture and the low increase rate of the fluorescence intensity in proportion to the amount of the template DNA.

From the results of Example 1 and Comparative Examples 1 and 2, the quantitative determination can be conducted precisely by utilizing the fluorescence 2,4-bis(4-N,N-dimethylaminophenyl-6-methyl)pyrylium iodide not influenced by the higher-order structured product formed from the primers. This result is well consistent with the result of EB staining of agarose gel electrophoresis of the reaction solutions in Example 1. In EB staining of the agarose gel, however, the fluorescence saturated at the template DNA levels of 500 pg and 100 pg, apparently showing the presence of the same quantity of the amplification products of the template DNA levels of 100 pg or higher. On the other hand, in the detection with 2,4-bis(4-N,N-dimethylaminophenyl)-6-methylpyrylium salt, the fluorescence intensity does not saturate even at the levels in which EB fluorescence saturates, thereby, quantitative determination being practicable.

Example 2 [Measurement of bacterial cell number by PCR employing a microplate]

An overnight culture (2×YT medium) of E. coli JM109 strain was added to soil. From the soil, DNA was extracted in a conventional manner, and subjected to PCR targeting 16S rRNA gene of E. coli as the template, to determine the number of bacterial cells by PCR-MPN method.

E. coli was added to the soil to give cell numbers per gram of soil of about 10^7, about 10^6, about 10^5, and about 10^4 (corresponding to Samples A, B, C, and D in Fig. 4, respectively), and the soil samples were stirred. One gram of the soil sample containing E. coli was respectively suspended in 0.5 ml of 1M phosphate buffer solution (pH 8.0). To the suspension, 0.05 ml of 10% SDS solution was added. The mixture, after being sufficiently stirred, was left standing at 70 °C for one hour. The suspension was mixed with a vortex mixer for complete cell lysis. To the lysate, an equal amount of a phenol-chloroform mixture was mixed. After centrifugation, the upper layer was collected. Thereto, twice the amount of ethanol was added to recover DNA as the precipitate. This precipitate was dissolved in 100 μl of a TE buffer solution (pH 8.0) to form a template DNA solution for PCR.

The following two PCR primers selective for 16S rRNA gene of E. coli were employed.

Primer 1: 5' AGAGTTTGATCCTGGCTCAG 3' (Sequence No. 3)

Primer 2: 5' AACCCAACATCTCACGACAC 3' (Sequence No. 4)

These primers were synthesized by means of Synthesizer 381A made by ABI Co. The reagents and the techniques for the synthesis were based on the protocol of ABI Co.

PCR was conducted with the extracted DNA as the template and the primers under the same conditions as in Example 1. The PCR was conducted in the wells (U-bottomed) of a 96-well microtiter plate (Falcon assay plate 3911 made by Becton-Dickinson Inc.). Each well of the microplate contains preliminarily applied and dried 1 μl of a solution of 2,4-bis(N,N-dimethylaminophenyl)-6-methylpyrylium iodide in acetonitrile of the same concentration as in Example 1. Starting from the DNA solution (the extracted DNA solved in 100 μl), eight successive 10-fold dilutions of the template DNA (10^{-1} to 10^{-8}) were prepared and each dilution (1 μl) was distributed in five wells in one line as shown in Fig. 4 for the PCR reaction. The PCR reaction apparatus was Model PTC-100-96 made by MJ Research Inc.

After the PCR reaction, 5 μl of acetonitrile was added to each of the wells on the microplate. Each mixture was agitated sufficiently, and was left standing for 5 minutes. Then the microplate was set on a Millipore Fluorescence Apparatus (CytoFluor 2350), and the fluorescence was measured with the same filters employed in Example 1. Fig. 5 shows the results. The wells producing fluorescence of twice or more that of the blank wells were regarded to contain a PCR amplification product (shadowed wells in Fig. 5). From the results, the numbers of E. coli in the respective soil samples were estimated to be 4.9×10^5, 7.9×10^5, 2.2×10^5, and 7.9×10^3 by reference to the table for MPN.

Comparative Example 3

The numbers of E. coli cells in the same soil samples prepared in Example 2 were measured by plate count method in which a predetermined amount of the sample was inoculated in an E coli culture plate and the number of colonies were measured as the viable cell number. The results agreed well with the results obtained in Example 2 as shown in Fig. 6.

From the results of Example 2 and Comparative Example 3, the PCR method of the present invention enables measurement of the number of the cells with simple operations giving well agreed results with that obtained by conventional plate count method.

Example 3 [Determination of template DNA by PCR method employing a microplate]

In each of the wells of a microplate as used in Example 2, 1 μl of 2,4-bis(4-N,N-dimethylaminophenyl)-6-methylpyrylium iodide solution in acetonitrile and the primers (two kinds of primers, 10 pmoles) as used in Example 2 were applied and dried.

Separately, from 2 ml of an overnight culture of E. coli, DNA of E. coli was extracted in the same manner as in Example 2, and the extract was diluted 100-fold to obtain a template DNA solution.

This template DNA solution was serially diluted in the same manner as in Example 2. The dilutions were added by 1 μl each to the wells of the microplate as shown in Fig. 5. To each of the wells, the PCR reaction solution was added which contains one Ampli Wax PCR Gem 100 (made by Perkin-Elmer) and the components below:

10× buffer:	5 μl
dNTPs:	5 μl
Taq DNA polymerase:	0.5 unit

Further, to each of the wells, sterilized water was added to the total volume of 50 μl. Then PCR was carried out with a PCR apparatus, Model PTC-100-96 (made by MJ Research Inc.), under the same conditions as in Example 1.

After the PCR reaction, 5 μl of acetonitrile was added to each of the wells on the microplate. Each of the mixture was agitated sufficiently, and was left standing for 5 minutes. Then the microplate was set on a Millipore Fluorescence Apparatus (Cytofluor 2350), and the fluorescence was measured with the same filters employed in Example 1. Fig. 7 shows the results. The wells producing fluorescence of twice or more that of the blank wells were regarded to contain the PCR amplification product (shadowed wells in Fig. 5). From the results, the number of the template DNA molecules was estimated to be 3.5×10^7 by reference to the table for MPN. In consideration of the initial dilution by the factor of 100, the number of the template DNA molecules was estimated to be 3.5×10^9.

Comparative Example 4

The number of the bacterial cells in the 2 ml of E. coli culture used in Example 3 was determined by plate count method. Consequently, the number was found to be 4×10^9, which agrees approximately with the results obtained in Example 3.

The desirable dilution degree of a DNA template and the desirable concentration range thereof for MPN detection can be decided, and the sample concentration can be set according to the results of Examples 2 and 3. Therefore calibration curves can be prepared for the number of the cells, the copy number, and the amount of template DNA.

Example 4 [Detection kit for cancer-specific gene by PCR]

(1) Extraction of mRNA:

Two tissue samples of 5 mm cube were taken out respectively from two cancer-suspected sites of the large intestine of a patient by biopsy. The samples are hereinafter referred to as "Tissue A" and "Tissue B". From the tissue samples, mRNA was extracted by a conventional manner as follows (by reference to "Shin Seikagaku Jikken Koza (New Library of Experiments in Biochemistry)" vol. 2, Nucleic acid I, page 48): 2 ml of D Solution (4M guanidine thiocyanate, 25 mM sodium citrate (pH 7.0), 0.5% sodium N-lauroylsarcosinate, and 0.1M 2-mercaptoethanol) was added to each of the tissue samples, and the tissue was immediately homogenized in a sterilized tube by means of Polytron three times each for 10 seconds. Thereto were added successively 0.2 ml of 2M sodium acetate (pH 4), 2 ml of water-saturated phenol, and 0.4 ml of chloroform-isopentyl alcohol (49:1 in volume ratio) with sufficient stirring at each addition. The mixture was shaken by means of a Vortex mixer for 10 seconds, cooled with ice for 15 minutes, and subjected to centrifugation at 4 °C at 10000 × g for 20 minutes. After the centrifugation, to the aqueous layer, an equal amount of isopropyl alcohol was added, and the mixture was left standing at -20 °C overnight. The mixture was then centrifuged as above and 0.6 ml of D Solution was added to dissolve the precipitate. Thereto an equal amount of isopropyl alcohol was added. The mixture was cooled at -20 °C for one hour, and centrifuged at 4 °C at 10000 × g for 20 minutes. The obtained precipitate was suspended in 75% ethanol, and the suspension was centrifuged again at 10000 x g, at 4 °C for 20 minutes. The precipitate was dried as a crude RNA fraction, which was heated at 65 °C for 5 minutes, then cooled rapidly to room temperature, and was washed with an equal amount of a 2x TNEL buffer solution [20 mM Tris-hydrochloric acid buffer solution (pH 7.6), 0.5M sodium chloride, 1mM EDTA, and 0.1% sodium N-lauroylsarcosinate]. The washed crude RNA fraction was applied to an oligo(dT)-cellulose column made by Pharmacia) equilibrated with a TNEL buffer solution, and mRNA was eluted with an extraction solution (TNEL buffer solution without 0.5M sodium chloride).

(2) Preparation of cDNA:

Double-stranded cDNA was prepared from the above mRNA by means of TimeSaver™ cDNA Synthesis Kit.

(3) Constitution of quantitative determination kit:

The primers used for detection of large intestine cancer have following nucleotide sequences.

Primer 1: 5' GACTCTGGAGTGAGAATCATA 3' (Sequence No. 5)

Primer 2: 5' ATCCAATCACCCACATGCATT 3' (Sequence No. 6)

To the bottom of Vessel 1 (an Epfendorf tube) as shown in Figs. 9A to 9C, Primer 1 and Primer 2 were applied to an amount of 10 pmoles respectively. In the container 2 prepared from paraffin-coated paper, the components below were packed.

dNTPs:	5 μl
Taq polymerase:	0.5 unit
150 μg/ml 2,4-bis(4-N,N-dimethylaminophenyl)-6-methylpyrylium iodide solution in acetonitrile:	1 μl
10× buffer:	5 μl
Distilled water:	39 μl
(freeze stored)	

The container 2 was set in the vessel 1 as shown in Fig. 9B to make up a kit. The cDNA samples obtained above from each of the aforementioned Tissue A and Tissue B were serially diluted, 1-fold (no dilution), 2-fold, 10-fold, 50-fold, 100-fold, 1000-fold, and 10000-fold. Each dilution (including non-diluted sample) was injected with a Pipetman to the bottom of vessel 1 of the kit, breaking through the paraffin-coated paper container 2. After the injection of cDNA, the vessels were centrifuged to transfer the contents of the container 2 completely to the reaction zone 5 containing the primers. After confirming the complete transfer, the broken empty containers 2 were taken out from the vessel 1. Thereto, one Ampli Wax, PCR Gen 100 (made by Perkin-Elmer Co.) was added.

(4) Practice of PCR, and detection:

PCR was conducted in the same manner as in Example 1. The samples in the vessels were subjected to fluorescence measurement without further dilution. The results regarding Tissue A are shown in Fig. 8. The target gene amplification was observed with Tissue A, and the amplification product was formed quantitatively corresponding to the dilution degree of the template DNA. On the contrary, no fluorescence was observed with Tissue B.

(5) Examination by electrophoresis:

After the detection, DNA was recovered from the samples by precipitation with ethanol. The recovered matter was examined by agarose gel electrophoresis. The DNA from Tissue A gave a band at 317 bp at the 1-fold (not diluted) to 1000-fold dilutions, whereas no band was observed with Tissue A at 10000-fold dilution, the blank samples containing primers only, and the samples derived from Tissue B.

Comparative Example 5

The above Tissue A and Tissue B were examined by a conventional culture method. Tissue A exhibited multiplication, and was diagnosed as a malignant cancer, whereas Tissue B did not exhibit multiplication, and was diagnosed as benign polyp.

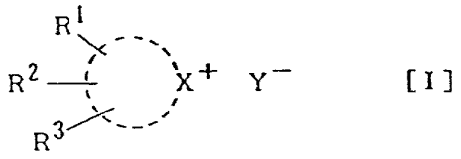
According to the present invention, the dye compounds which reacts selectively with double-stranded nucleic acid is used for detection of the PCR amplification products, enabling simplification of detection, quantitative determination, and measurement of the number of the cells with precision.

A PCR amplification product is detected, in quantitative determination of nucleic acid and measurement of the number of bacterial cells or specific genes, by addition of a dye compound which does not fluoresce in the free state but fluoresces in the bonded state to a double-stranded nucleic acid.

Claims

1. A method for quantitative nucleic acid determination, comprising conducting PCR of a nucleic acid sample with a primer set required for amplification of a specific sequence region of a target nucleic acid; reacting a double-stranded amplification product which will be formed when the target nucleic acid is present in the sample with a dye compound which does not fluoresce in the free state but fluoresces in the bonded state to double-stranded nucleic acid, and measuring intensity of the fluorescence to quantitatively determine the target nucleic acid in the nucleic acid sample.

2. The method for quantitative nucleic acid determination according to claim 1, wherein the dye compound is represented by the general formula [I] below:



[I]

where

represents a heterocycle where X is O, S, Se, or Te, the heterocycle including a five-membered and six-membered ring such as a pyrylium ring and pyrylium-analogous ring;

R^1 and R^2 are independently a hydrogen atom, a halogen atom, a sulfonate group, an amino group, a styryl group, a nitro group, a hydroxyl group, a carboxyl group, a cyano group, a substituted or unsubstituted lower alkyl group, a substituted or unsubstituted aryl group, a substituted or unsubstituted

lower aralkyl group, or a substituted or unsubstituted cycloalkyl group;
R^3 is a group of -A or -L-A wherein L is $-L^1-$, $-L^2-L^3-$, or $-L^4-L^5-L^6-$, L^1 to L^6 being independently -(CH = CH)-, a bivalent group derived from a substituted or unsubstituted aryl group, a substituted or unsubstituted alkylene group, or $-CH=R^4-$ (R^4 being a cyclic structure having an oxo group); A is a substituted or unsubstituted aryl group, or $-CH=R^5$ (R^5 being a substituted or unsubstituted heterocycle, a substituted or unsubstituted cycloalkyl group, or a substituted or unsubstituted aromatic ring);
in the pyrylium ring or a pyrylium-analogous ring containing X, the hydrogen atom bonded to the carbon atom which is not linked to R^1, R^2, or R^3 may be substituted by a halogen atom, a sulfonate group, an amino group, a styryl group, a nitro group, a hydroxyl group, a carboxyl group, a cyano group, a substituted or unsubstituted lower alkyl group, a substituted or unsubstituted aryl group, or a substituted or unsubstituted lower aralkyl group; and
Y^- is an anion.

3. The method for quantitative nucleic acid determination according to claim 2, wherein the dye compound represented by the general formula [I] is a 2,4-bis(4-N,N-dimethylaminophenyl)-6-methylpyrylium salt, or a 2,4-bis(4-N,N-dimethylaminophenyl)-6-methylthiopyrylium salt.

4. The method for quantitative nucleic acid determination according to any one of claims 1 to 3, wherein the dye compound is insoluble in water; PCR is conducted in an aqueous reaction system containing the dye compound and an aqueous medium; and a solvent is added to the reaction system after the PCR to dissolve the dye compound thus enabling the reaction with the amplification product.

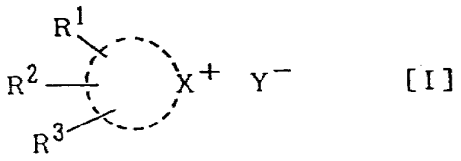
5. The method for quantitative nucleic acid determination according to any one of claims 1 to 4, wherein the amplification product has a chain length of not less than 100 base pairs.

6. The method for quantitative nucleic acid determination according to any one of claims 1 to 5, wherein the primer has a chain length of 30 bases or less.

7. A kit for quantitative nucleic acid determination, comprising a reactor containing a required amount of a dye compound in a reaction chamber for PCR, the dye compound not producing fluorescence in the free state but producing fluorescence in the bonded state to double-stranded nucleic acid.

8. The kit for quantitative nucleic acid determination according to claim 7, wherein the reactor further contains in the reaction chamber a required amount of a primer set which is necessary for the PCR amplification of a specific sequence region of a target nucleic acid.

9. The kit for quantitative determination of a nucleic acid according to claim 7 or 8, wherein the dye compound is represented by the general formula [I] below:



[I]

where

represents a heterocycle, and X is O, S, Se, or Te, the heterocycle including a five-membered and six-membered ring such as a pyrylium ring and pyrylium-analogous ring;
R^1 and R^2 are independently a hydrogen atom, a halogen atom, a sulfonate group, an amino group, a styryl group, a nitro group, a hydroxyl group, a carboxyl group, a cyano group, a substituted or unsubstituted lower alkyl group, a substituted or unsubstituted aryl group, a substituted or unsubstituted

lower aralkyl group, or a substituted or unsubstituted cycloalkyl group;
R^3 is a group of -A or -L-A wherein L is $-L^1-$, $-L^2-L^3-$, or $-L^4-L^5-L^6-$, L^1 to L^6 being independently -(CH=CH)-, a bivalent group derived from a substituted or unsubstituted aryl group, a substituted or unsubstituted alkylene group, or $-CH=R^4-$ (R^4 being a cyclic structure having an oxo group); A is a substituted or unsubstituted aryl group, or $-CH=R^5$ (R^5 being a substituted or unsubstituted heterocycle, a substituted or unsubstituted cycloalkyl group, or a substituted or unsubstituted aromatic ring);
in the pyrylium ring or a pyrylium-analogous ring containing X, the hydrogen atom bonded to the carbon atom, which is not linked to R^1, R^2, or R^3, may be substituted by a halogen atom, a sulfonate group, an amino group, a styryl group, a nitro group, a hydroxyl group, a carboxyl group, a cyano group, a substituted or unsubstituted lower alkyl group, a substituted or unsubstituted aryl group, or a substituted or unsubstituted lower aralkyl group; and
Y^- is an anion.

10. The kit for quantitative nucleic acid determination according to claim 9, wherein the dye compound represented by the general formula [I] is a 2,4-bis(4-N,N-dimethylaminophenyl)-6-methylpyrylium salt, or a 2,4-bis(4-N,N-dimethylaminophenyl)-6-methylthiopyrylium salt.

11. The kit for quantitative nucleic acid determination according to any of claims 7 to 10, wherein the dye compound is water-insoluble, and a solvent is provided to dissolve the dye compound thus enabling reaction of the dye compound with the amplification product in the reaction chamber after the PCR.

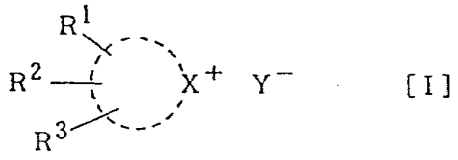
12. A kit for quantitative nucleic acid determination, comprising a reaction chamber for PCR and a reagent chamber separated therefrom, the reagent chamber containing a required amount of a dye compound which does not fluoresce in the free state but fluoresces in the bonded state to double-stranded nucleic acid, the dye compound in the reagent chamber being provided so as to be transferable to the reaction chamber.

13. The kit for quantitative nucleic acid determination according to claim 12, wherein the reagent chamber contains also a reagent for the PCR.

14. The kit for quantitative nucleic acid determination according to claim 13, wherein the reagent chamber is divided into several chambers, and a dye compound-containing reagent chamber and a PCR reagents-containing reagent chamber.

15. The kit for quantitative nucleic acid determination according to any of claims 12 to 14, wherein a necessary amount of a primer set required for PCR amplification of a specific sequence region of a target nucleic acid is contained in the reaction chamber.

16. The kit for quantitative nucleic acid determination according to any of claims 12 to 15, wherein the dye compound is represented by the general formula [I] below:



[I]

where

represents a heterocycle, and X is O, S, Se, or Te, the heterocycle including a five-membered and six-membered ring such as a pyrylium ring and pyrylium-analogous ring;

R^1 and R^2 are independently a hydrogen atom, a halogen atom, a sulfonate group, an amino group, a styryl group, a nitro group, a hydroxyl group, a carboxyl group, a cyano group, a substituted or unsubstituted lower alkyl group, a substituted or unsubstituted aryl group, a substituted or unsubstituted lower aralkyl group, or a substituted or unsubstituted cycloalkyl group;
R^3 is a group of -A or -L-A wherein L is $-L^1-$, $-L^2-L^3-$, or $-L^4-L^5-L^6-$, L^1 to L^6 being independently -(CH=CH)-, a bivalent group derived from a substituted or unsubstituted aryl group, a substituted or unsubstituted alkylene group, or $-CH=R^4-$ (R^4 being a cyclic structure having an oxo group); A is a substituted or unsubstituted aryl group, or $-CH=R^5$ (R^5 being a substituted or unsubstituted heterocycle, a substituted or unsubstituted cycloalkyl group, or a substituted or unsubstituted aromatic ring);
in the pyrylium ring or a pyrylium-analogous ring containing X, the hydrogen atom bonded to the carbon atom, which is not linked to R^1, R^2, or R^3, may be substituted by a halogen atom, a sulfonate group, an amino group, a styryl group, a nitro group, a hydroxyl group, a carboxyl group, a cyano group, a substituted or unsubstituted lower alkyl group, a substituted or unsubstituted aryl group, or a substituted or unsubstituted lower aralkyl group; and
Y^- is an anion.

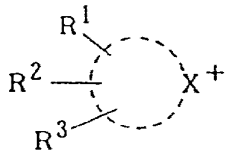
17. The kit for quantitative determination of a nucleic acid according to claim 16, wherein the dye compound represented by the general formula [I] is a 2,4-bis(N,N-dimethylaminophenyl)-6-methylpyrylium salt, or a 2,4-bis(N,N-dimethylaminophenyl)-6-methylthiopyrylium salt.

18. The kit for quantitative nucleic acid determination according to any of claims 12 to 17, wherein the dye compound is water-insoluble, and a solvent is provided to dissolve the dye compound, thus enabling the reaction of the dye compound with the amplification product in the reaction chamber after the PCR.

19. The kit for quantitative nucleic acid determination according to any of claims 12 to 17, wherein a solution of the dye compound is placed in the reagent chamber.

20. A method for measurement of number of target microorganism or cells, number of specific gene, or copy number of a specific gene, comprising extracting nucleic acid from a sample containing a microorganism or cells to be detected; preparing a serial dilution of the extracted nucleic acid; conducting PCR with the sample dilutions to amplify a sequence which is characteristic of the microorganism or cells; reacting the resulting amplification product of double-stranded nucleic acid with a dye compound which does not fluoresce in the free state but fluoresces in the bonded state to the double-stranded nucleic acid; measuring intensity of the fluorescence; and deriving the number of the microorganism or cells, the specified genes, or copies of the specified genes in the sample on the basis of the dilution rate at which fluorescence is observed.

21. The method for measurement according to claim 20, wherein the dye compound is represented by the general formula [I] below:



[I]

where

represents a heterocycle, and X is O, S, Se, or Te, the heterocycle including a five-membered and six-membered ring such as a pyrylium ring and pyrylium-analogous ring;
R^1 and R^2 are independently a hydrogen atom, a halogen atom, a sulfonate group, an amino group, a styryl group, a nitro group, a hydroxyl group, a carboxyl group, a cyano group, a substituted or

unsubstituted lower alkyl group, a substituted or unsubstituted aryl group, a substituted or unsubstituted lower aralkyl group, or a substituted or unsubstituted cycloalkyl group;
R^3 is a group of -A or -L-A wherein L is $-L^1-$, $-L^2-L^3-$, or $-L^4-L^5-L^6-$, L^1 to L^6 being independently -(CH=CH)-, a bivalent group derived from a substituted or unsubstituted aryl group, a substituted or unsubstituted alkylene group, or $-CH=R^4-$ (R^4 being a cyclic structure having an oxo group); A is a substituted or unsubstituted aryl group, or $-CH=R^5$ (R^5 being a substituted or unsubstituted heterocycle, a substituted or unsubstituted cycloalkyl group, or a substituted or unsubstituted aromatic ring);
in the pyrylium ring or a pyrylium-analogous ring containing X, the hydrogen atom bonded to the carbon atom, which is not linked to R^1, R^2, or R^3, may be substituted by a halogen atom, a sulfonate group, an amino group, a styryl group, a nitro group, a hydroxyl group, a carboxyl group, a cyano group, a substituted or unsubstituted lower alkyl group, a substituted or unsubstituted aryl group, or a substituted or unsubstituted lower aralkyl group; and
Y^- is an anion.

22. The method for measurement according to claim 21, wherein the dye compound represented by the general formula [I] is a 2,4-bis(4-N,N-dimethylaminophenyl)-6-methylpyrylium salt, or a 2,4-bis(4-N,N-dimethylaminophenyl)-6-methylthiopyrylium salt.

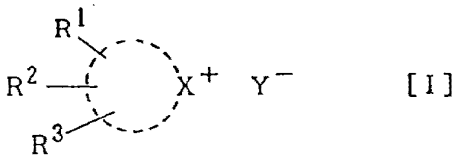
23. The method for measurement according to any of claims 20 to 22, wherein the dye compound is water-insoluble, and the PCR is conducted in a reaction system containing the water-insoluble dye compound and an aqueous medium, a solvent is added after the PCR to the reaction system to dissolve the dye compound thus enabling the reaction of the dye compound with the amplification product.

24. The method for measurement according to any of claims 20 to 23, wherein the amplification product has a chain length of not less than 100 base pairs.

25. The method for measurement according to any of claims 20 to 24, wherein the primer has a chain length of 30 bases or less.

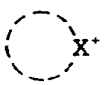
26. A kit for measurement of number of target microorganism or cells, number of specific genes, or copy number of the specific genes, comprising a reactor having a plurality of reaction chambers for PCR each containing therein a required amount of a dye compound which does not fluoresce in the free state but fluoresces in the bonded state to double-stranded nucleic acid; the reaction chambers being employed for practicing serial dilution of a nucleic acid extract obtained from a sample containing a microorganism or cells to be detected, and employed for the PCR for each dilution to amplify a sequence which is characteristic of the microorganism or cells to be detected.

27. The kit according to claim 26, wherein the dye compound is represented by the general formula [I] below:



[I]

where



represents a heterocycle, and X is O, S, Se, or Te, the heterocycle including a five-membered and six-membered ring such as a pyrylium ring and pyrylium-analogous ring;
R^1 and R^2 are independently a hydrogen atom, a halogen atom, a sulfonate group, an amino group, a

styryl group, a nitro group, a hydroxyl group, a carboxyl group, a cyano group, a substituted or unsubstituted lower alkyl group, a substituted or unsubstituted aryl group, a substituted or unsubstituted lower aralkyl group, or a substituted or unsubstituted cycloalkyl group;
R^3 is a group of -A or -L-A wherein L is $-L^1-$, $-L^2-L^3-$, or $-L^4-L^5-L^6-$, L^1 to L^6 being independently -(CH=CH)-, a bivalent group derived from a substituted or unsubstituted aryl group, a substituted or unsubstituted alkylene group, or $-CH=R^4-$ (R^4 being a cyclic structure having an oxo group); A is a substituted or unsubstituted aryl group, or $-CH=R^5$ (R^5 being a substituted or unsubstituted heterocycle, a substituted or unsubstituted cycloalkyl group, or a substituted or unsubstituted aromatic ring);
in the pyrylium ring or a pyrylium-analogous ring containing X, the hydrogen atom bonded to the carbon atom, which is not linked to R^1, R^2, or R^3, may be substituted by a halogen atom, a sulfonate group, an amino group, a styryl group, a nitro group, a hydroxyl group, a carboxyl group, a cyano group, a substituted or unsubstituted lower alkyl group, a substituted or unsubstituted aryl group, or a substituted or unsubstituted lower aralkyl group; and
Y^- is an anion.

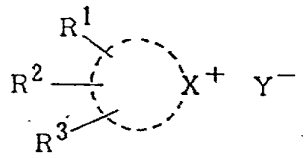
28. The kit according to claim 26 or 27, wherein the dye compound represented by the general formula [I] is a 2,4-bis(4-N,N-dimethylaminophenyl)-6-methylpyrylium salt, or a 2,4-bis(4-N,N-dimethylaminophenyl)-6-methylthiopyrylium salt.

29. The kit according to any of claims 26 to 28, wherein the dye compound is water-insoluble, and a solvent is provided for dissolving the dye compound thus enabling the reaction of the dye compound with the amplification product in the reaction chamber.

30. The kit according to any of claims 26 to 29, wherein the reaction chamber further contains a primer set for PCR amplification of a sequence characteristic of the microorganism or cells to be detected.

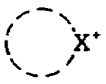
31. A method for quantitative nucleic acid determination, comprising conducting MPN-PCR of the target nucleic acid with a primer set required for amplification of a specific sequence region of the target nucleic acid; reacting a double-stranded amplification product which will be formed when the target nucleic acid is present with a dye compound which does not fluoresce in the free state but fluoresces in the bonded state to the double-stranded nucleic acid, and measuring intensity of the fluorescence to determine the target nucleic acid in a sample.

32. The method for quantitative nucleic acid determination according to claim 31, wherein the dye compound is represented by the general formula [I] below:



[I]

where



represents a heterocycle, and X is O, S, Se, or Te, the heterocycle including a five-membered and six-membered ring such as a pyrylium ring and pyrylium-analogous ring;
R^1 and R^2 are independently a hydrogen atom, a halogen atom, a sulfonate group, an amino group, a styryl group, a nitro group, a hydroxyl group, a carboxyl group, a cyano group, a substituted or unsubstituted lower alkyl group, a substituted or unsubstituted aryl group, a substituted or unsubstituted lower aralkyl group, or a substituted or unsubstituted cycloalkyl group;
R^3 is a group of -A or -L-A wherein L is $-L^1-$, $-L^2-L^3-$, or $-L^4-L^5-L^6-$, L^1 to L^6 being independently -(CH=CH)-, a bivalent group derived from a substituted or unsubstituted aryl group, a substituted or

unsubstituted alkylene group, or -CH = R^4- (R^4 being a cyclic structure having an oxo group); A is a substituted or unsubstituted aryl group, or -CH = R^5 (R^5 being a substituted or unsubstituted heterocycle, a substituted or unsubstituted cycloalkyl group, or a substituted or unsubstituted aromatic ring);
in the pyrylium ring or a pyrylium-analogous ring containing X, the hydrogen atom bonded to the carbon atom, which is not linked to R^1, R^2, or R^3, may be substituted by a halogen atom, a sulfonate group, an amino group, a styryl group, a nitro group, a hydroxyl group, a carboxyl group, a cyano group, a substituted or unsubstituted lower alkyl group, a substituted or unsubstituted aryl group, or a substituted or unsubstituted lower aralkyl group; and
Y^- is an anion.

33. The method for quantitative nucleic acid determination according to claim 32, wherein the dye compound represented by the general formula [I] is a 2,4-bis(4-N,N-dimethylaminophenyl)-6-methylpyrylium salt, or a 2,4-bis(4-N,N-dimethylaminophenyl)-6-methylthiopyrylium salt.

34. The method for quantitative nucleic acid determination according to any one of claims 31 to 33, wherein the dye compound is insoluble in water; the MPN-PCR is conducted in an aqueous reaction system containing the dye compound and an aqueous medium; and a solvent is added to the reaction system after the MPN-PCR to dissolve the dye compound thus enabling the reaction with the amplification product.

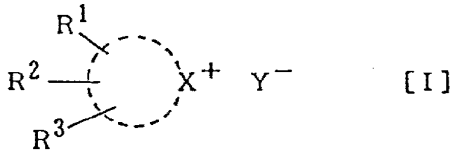
35. The method for quantitative nucleic acid determination according to any one of claims 31 to 34, wherein the amplification product has a chain length of not less than 100 base pairs.

36. The method for quantitative nucleic acid determination according to any of claims 31 to 35, wherein the primer has a chain length of 30 bases or less.

37. A kit for quantitative nucleic acid determination, comprising a reactor containing a required amount of a dye compound in a reaction chamber for MPN-PCR, the dye compound not producing fluorescence in the free state but producing fluorescence in the bonded state to double-stranded nucleic acid.

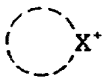
38. The kit for quantitative determination of nucleic acid according to claim 37, wherein the reactor further contains in the reaction chamber a required amount of a primer set which is necessary for MPN-PCR amplification of a specific sequence region of target nucleic acid.

39. The kit for quantitative determination of nucleic acid according to claim 37 or 38, wherein the dye compound is represented by the general formula [I] below:



[I]

where



represents a heterocycle, and X is O, S, Se, or Te, the heterocycle including a five-membered and six-membered ring such as a pyrylium ring and pyrylium-analogous ring;
R^1 and R^2 are independently a hydrogen atom, a halogen atom, a sulfonate group, an amino group, a styryl group, a nitro group, a hydroxyl group, a carboxyl group, a cyano group, a substituted or unsubstituted lower alkyl group, a substituted or unsubstituted aryl group, a substituted or unsubstituted lower aralkyl group, or a substituted or unsubstituted cycloalkyl group;

R^3 is a group of -A or -L-A wherein L is $-L^1-$, $-L^2-L^3-$, or $-L^4-L^5-L^6-$, L^1 to L^6 being independently -(CH=CH)-, a bivalent group derived from a substituted or unsubstituted aryl group, a substituted or unsubstituted alkylene group, or $-CH=R^4-$ (R^4 being a cyclic structure having an oxo group); A is a substituted or unsubstituted aryl group, or $-CH=R^5$ (R^5 being a substituted or unsubstituted heterocycle, a substituted or unsubstituted cycloalkyl group, or a substituted or unsubstituted aromatic ring);
in the pyrylium ring or a pyrylium-analogous ring containing X, the hydrogen atom bonded to the carbon atom, which is not linked to R^1, R^2, or R^3, may be substituted by a halogen atom, a sulfonate group, an amino group, a styryl group, a nitro group, a hydroxyl group, a carboxyl group, a cyano group, a substituted or unsubstituted lower alkyl group, a substituted or unsubstituted aryl group, or a substituted or unsubstituted lower aralkyl group; and
Y^- is an anion.

40. The kit for quantitative determination of nucleic acid according to claim 39, wherein the dye compound represented by the general formula [I] is a 2,4-bis(4-N,N-dimethylaminophenyl)-6-methylpyrylium salt, or a 2,4-bis(4-N,N-dimethylaminophenyl)-6-methylthiopyrylium salt.

41. The kit for quantitative determination of a nucleic acid according to any of claims 37 to 40, wherein the dye compound is water-insoluble, and a solvent is provided to dissolve the dye compound thus enabling reaction of the dye compound with the amplification product in the reaction chamber after the MPN-PCR.

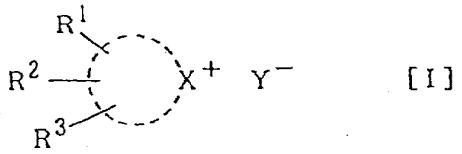
42. A kit for quantitative determination of nucleic acid, comprising a reaction chamber for MPN-PCR and a reagent chamber separated therefrom, the reagent chamber containing a required amount of a dye compound which does not fluoresce in the free state but fluoresces in the bonded state to a double-stranded nucleic acid, the dye compound in the reagent chamber being provided so as to be transferable to the reaction chamber.

43. The kit for quantitative determination of nucleic acid according to claim 42, wherein the reagent chamber contains also reagents for the MPN-PCR.

44. The kit for quantitative determination of nucleic acid according to claim 43, wherein the reagent chamber is divided into several chambers, and a dye compound-containing reagent chamber and a MPN-PCR reagent-containing reagent chamber are provided.

45. The kit for quantitative determination of nucleic acid according to any of claims 42 to 44, wherein a necessary amount of a primer set required for MPN-PCR amplification of a specific sequence region of a target nucleic acid is contained further in the reaction chamber.

46. The kit for quantitative determination of nucleic acid according to any of claims 42 to 45, wherein the dye compound is represented by the general formula [I] below:



[I]

where

represents a heterocycle, and X is O, S, Se, or Te, the heterocycle including a five-membered and six-membered ring such as a pyrylium ring and pyrylium-analogous ring;
R^1 and R^2 are independently a hydrogen atom, a halogen atom, a sulfonate group, an amino group, a

styryl group, a nitro group, a hydroxyl group, a carboxyl group, a cyano group, a substituted or unsubstituted lower alkyl group, a substituted or unsubstituted aryl group, a substituted or unsubstituted lower aralkyl group, or a substituted or unsubstituted cycloalkyl group;
R^3 is a group of -A or -L-A wherein L is $-L^1-$, $-L^2-L^3-$, or $-L^4-L^5-L^6-$, L^1 to L^6 being independently -(CH = CH)-, a bivalent group derived from a substituted or unsubstituted aryl group, a substituted or unsubstituted alkylene group, or $-CH=R^4-$ (R^4 being a cyclic structure having an oxo group); A is a substituted or unsubstituted aryl group, or $-CH=R^5$ (R^5 being a substituted or unsubstituted heterocycle, a substituted or unsubstituted cycloalkyl group, or a substituted or unsubstituted aromatic ring);
in the pyrylium ring or a pyrylium-analogous ring containing X, the hydrogen atom bonded to the carbon atom, which is not linked to R^1, R^2, or R^3, may be substituted by a halogen atom, a sulfonate group, an amino group, a styryl group, a nitro group, a hydroxyl group, a carboxyl group, a cyano group, a substituted or unsubstituted lower alkyl group, a substituted or unsubstituted aryl group, or a substituted or unsubstituted lower aralkyl group; and
Y^- is an anion.

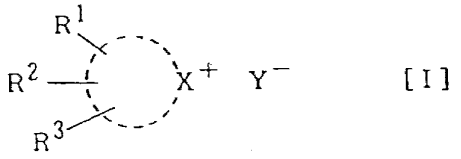
47. The kit for quantitative determination of nucleic acid according to claim 46, wherein the dye compound represented by the general formula [I] is a 2,4-bis(4-N,N-dimethylaminophenyl)-6-methylpyrylium salt, or a 2,4-bis(4-N,N-dimethylaminophenyl)-6-methylthiopyrylium salt.

48. The kit for quantitative determination of nucleic acid according to any of claims 42 to 47, wherein the dye compound is water-insoluble, and a solvent is provided to dissolve the dye compound thus enabling the reaction of the dye compound with the amplification product in the reaction chamber after the MPN-PCR.

49. The kit for quantitative determination of an MPN-PCR amplification product according to any of claims 42 to 47, wherein a solution of the dye compound is placed in the reagent chamber.

50. A method for measurement of number of target microorganism or cells, number of specific genes contained therein, or number of copies of the specific genes, comprising extracting nucleic acid from a sample containing a microorganism or cells to be detected; preparing a serially diluted sample solutions of the extracted nucleic acid; conducting MPN-PCR with the diluted samples to amplify a sequence which is characteristic of the microorganism or cells; reacting the resulting MPN-PCR amplification product containing a double-stranded nucleic acid with a dye compound which does not fluoresce in the free state but fluoresces in the bonded state to the double-stranded nucleic acid; measuring intensity of the fluorescence; and deriving the most probable number of the microorganism or cells, the specified genes, or copies of the specified genes from the highest dilution rate at which fluorescence is still observed.

51. The method for measurement according to claim 50, wherein the dye compound is represented by the general formula [I] below:



[I]

where

represents a heterocycle, and X is O, S, Se, or Te, the heterocycle including a five-membered and six-membered ring such as a pyrylium ring and pyrylium-analogous ring;

R^1 and R^2 are independently a hydrogen atom, a halogen atom, a sulfonate group, an amino group, a styryl group, a nitro group, a hydroxyl group, a carboxyl group, a cyano group, a substituted or unsubstituted lower alkyl group, a substituted or unsubstituted aryl group, a substituted or unsubstituted lower aralkyl group, or a substituted or unsubstituted cycloalkyl group;

R^3 is a group of -A or -L-A wherein L is $-L^1-$, $-L^2-L^3-$, or $-L^4-L^5-L^6-$, L^1 to L^6 being independently -(CH=CH)-, a bivalent group derived from a substituted or unsubstituted aryl group, a substituted or unsubstituted alkylene group, or $-CH=R^4-$ (R^4 being a cyclic structure having an oxo group); A is a substituted or unsubstituted aryl group, or $-CH=R^5$ (R^5 being a substituted or unsubstituted heterocycle, a substituted or unsubstituted cycloalkyl group, or a substituted or unsubstituted aromatic ring);

in the pyrylium ring or a pyrylium-analogous ring containing X, the hydrogen atom bonded to the carbon atom, which is not linked to R^1, R^2, or R^3, may be substituted by a halogen atom, a sulfonate group, an amino group, a styryl group, a nitro group, a hydroxyl group, a carboxyl group, a cyano group, a substituted or unsubstituted lower alkyl group, a substituted or unsubstituted aryl group, or a substituted or unsubstituted lower aralkyl group; and

Y^- is an anion.

52. The method for measurement according to claim 51, wherein the dye compound represented by the general formula [I] is a 2,4-bis(4-N,N-dimethylaminophenyl)-6-methylpyrylium salt, or a 2,4-bis(4-N,N-dimethylaminophenyl)-6-methylthiopyrylium salt.

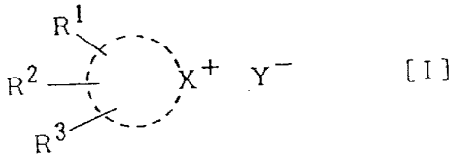
53. The method for measurement according to any of claims 50 to 52, wherein the dye compound is water-insoluble, and PCR is conducted in a reaction system containing the water-insoluble dye compound and an aqueous medium, a solvent is added after the MPN-PCR to the reaction system to dissolve the dye compound thus enabling reaction of the dye compound with the amplification product.

54. The method for measurement according to any of claims 50 to 53, wherein the amplification product has a chain length of not less than 100 base pairs.

55. The method for measurement according to any of claims 50 to 54, wherein the primer has a chain length of 30 bases or less.

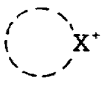
56. A kit for measurement of the number of target microorganism or cells, number of specific genes contained therein, or number of copies of the specific genes, comprising a reactor having a plurality of reaction chambers for MPN-PCR and containing therein a required amount of a dye compound which does not fluoresce in the free state but fluoresces in the bonded state to a double-stranded nucleic acid; the reaction chambers being employed for practicing serial dilution of a nucleic acid extract obtained form a sample containing microorganism or cells to be detected, and for the MPN-PCR for each dilution for amplification of a sequence which is characteristic of the microorganism or cells to be detected.

57. The kit according to claim 56, wherein the dye compound is represented by the general formula [I] below:



[I]

where



represents a heterocycle, and X is O, S, Se, or Te, the heterocycle including a five-membered and six-membered ring such as a pyrylium ring and pyrylium-analogous ring;
R^1 and R^2 are independently a hydrogen atom, a halogen atom, a sulfonate group, an amino group, a styryl group, a nitro group, a hydroxyl group, a carboxyl group, a cyano group, a substituted or unsubstituted lower alkyl group, a substituted or unsubstituted aryl group, a substituted or unsubstituted lower aralkyl group, or a substituted or unsubstituted cycloalkyl group;
R^3 is a group of -A or -L-A wherein L is $-L^1-$, $-L^2-L^3-$, or $-L^4-L^5-L^6-$, L^1 to L^6 being independently -(CH = CH)-, a bivalent group derived from a substituted or unsubstituted aryl group, a substituted or unsubstituted alkylene group, or $-CH=R^4-$ (R^4 being a cyclic structure having an oxo group); A is a substituted or unsubstituted aryl group, or $-CH=R^5$ (R^5 being a substituted or unsubstituted heterocycle, a substituted or unsubstituted cycloalkyl group, or a substituted or unsubstituted aromatic ring);
in the pyrylium ring or a pyrylium-analogous ring containing X, the hydrogen atom bonded to the carbon atom, which is not linked to R^1, R^2, or R^3, may be substituted by a halogen atom, a sulfonate group, an amino group, a styryl group, a nitro group, a hydroxyl group, a carboxyl group, a cyano group, a substituted or unsubstituted lower alkyl group, a substituted or unsubstituted aryl group, or a substituted or unsubstituted lower aralkyl group; and
Y^- is an anion.

58. The kit according to claim 56 or 57, wherein the dye compound represented by the general formula [I] is a 2,4-bis(4-N,N-dimethylaminophenyl)-6-methylpyrylium salt, or a 2,4-bis(4-N,N-dimethylaminophenyl)-6-methylthiopyrylium salt.

59. The kit according to any of claims 56 to 58, wherein the dye compound is water-insoluble, and a solvent is provided for dissolving the dye compound thus enabling reaction of the dye compound with the amplification product in the reaction chamber.

60. The kit according to any of claims 56 to 59, wherein the reaction chamber further contains a primer set for PCR amplification of a sequence characteristic of the microorganism or cells to be detected.

FIG. 1

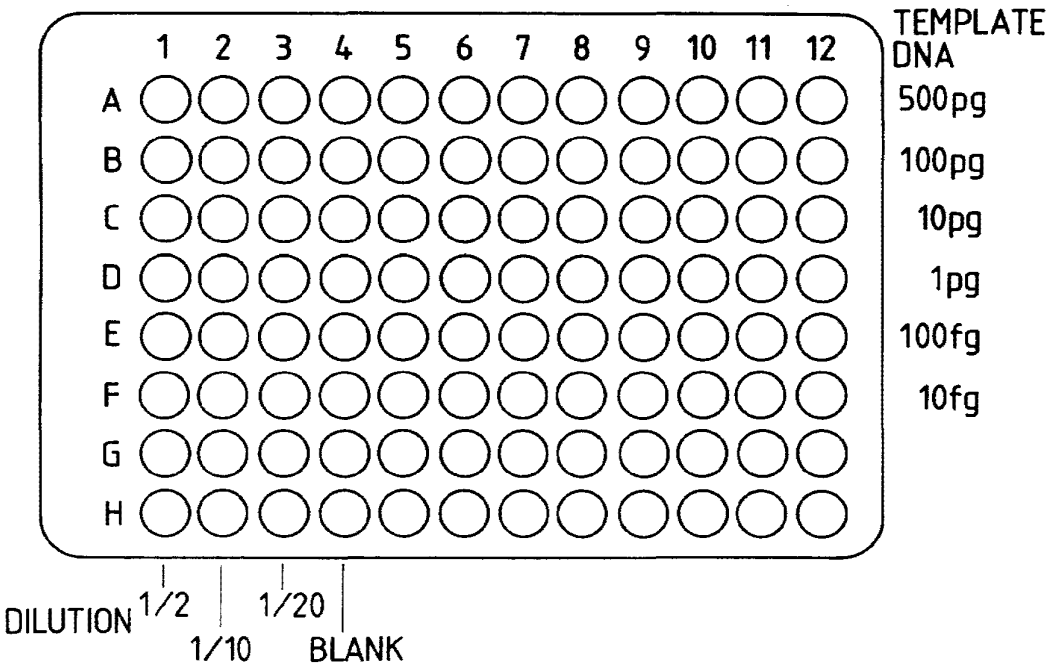

1 2 3 4 5 6 7 8 9 10 11 12
TEMPLATE DNA
A 500pg
B 100pg
C 10pg
D 1pg
E 100fg
F 10fg
G
H
DILUTION
1/2
1/10
1/20
BLANK

FIG. 2

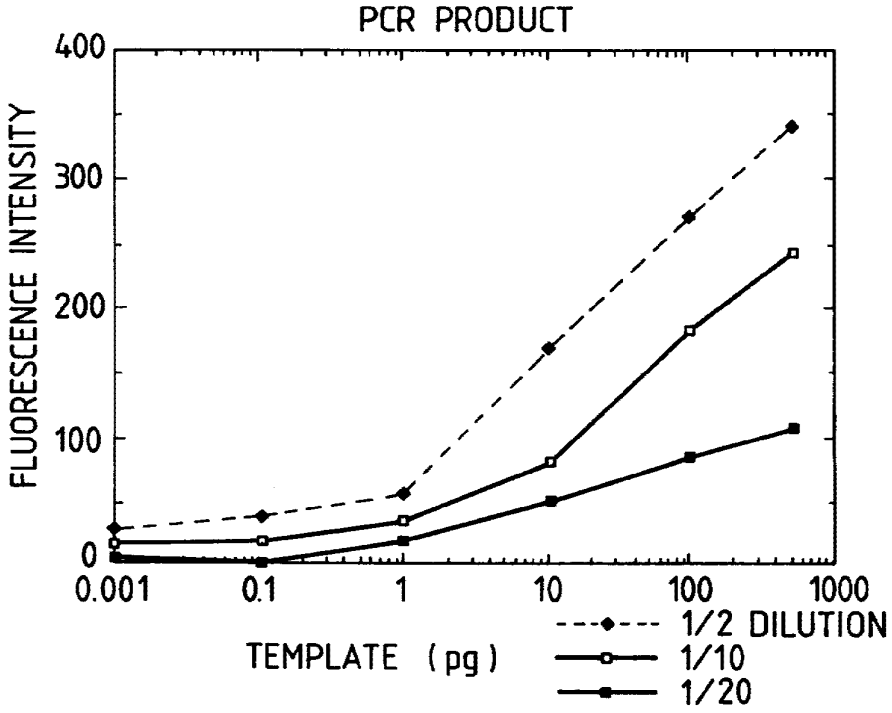

PCR PRODUCT
FLUORESCENCE INTENSITY
400
300
200
100
0
0.001
0.1
1
10
100
1000
TEMPLATE (pg)
1/2 DILUTION
1/10
1/20

FIG. 3

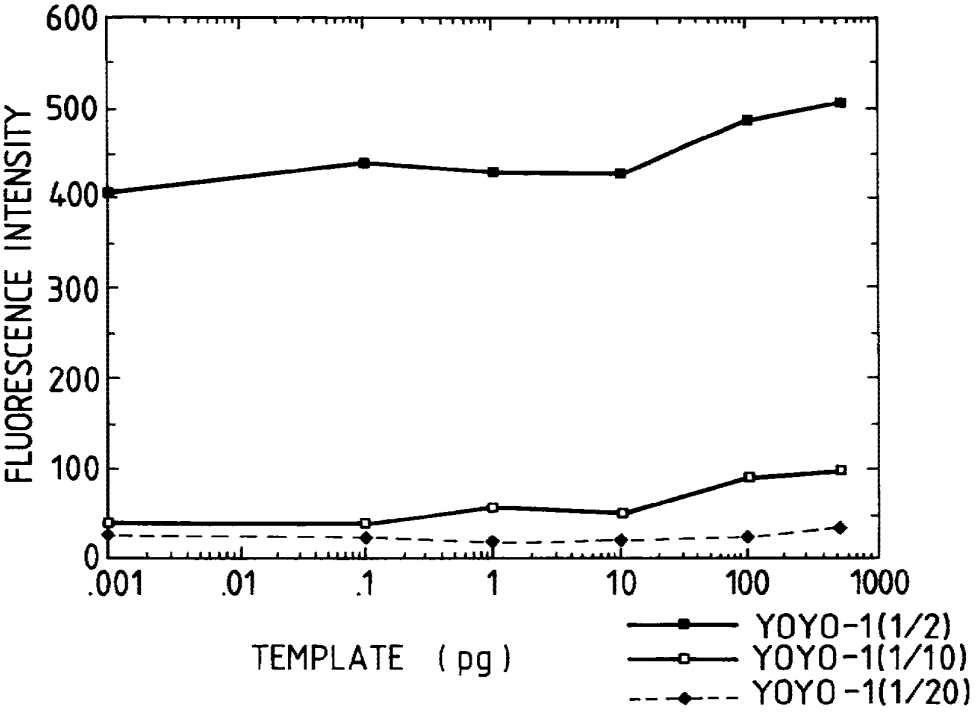

FLUORESCENCE INTENSITY
600
500
400
300
200
100
0
.001
.01
.1
1
10
100
1000
TEMPLATE (pg)
YOYO-1(1/2)
YOYO-1(1/10)
YOYO-1(1/20)

FIG. 4

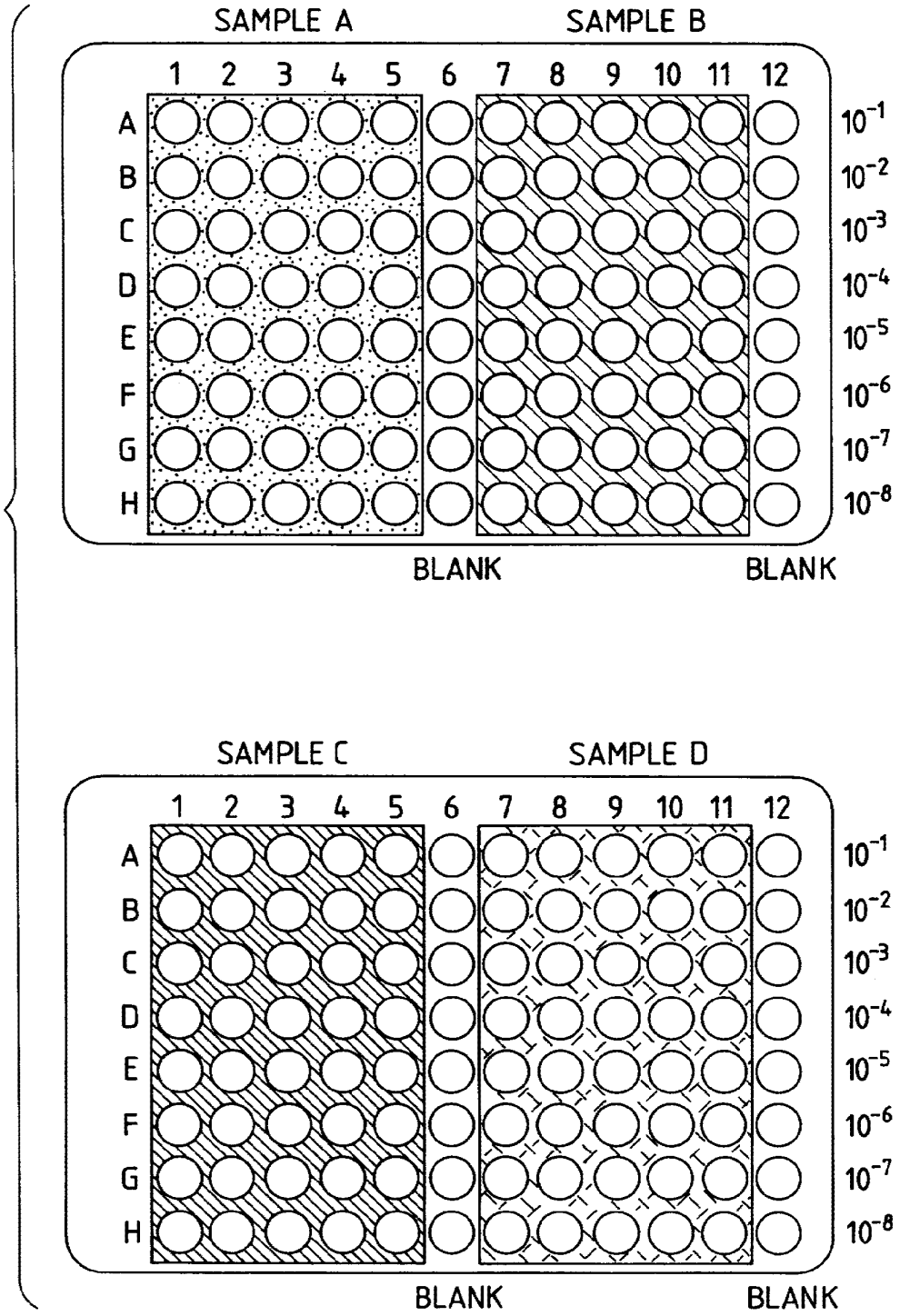
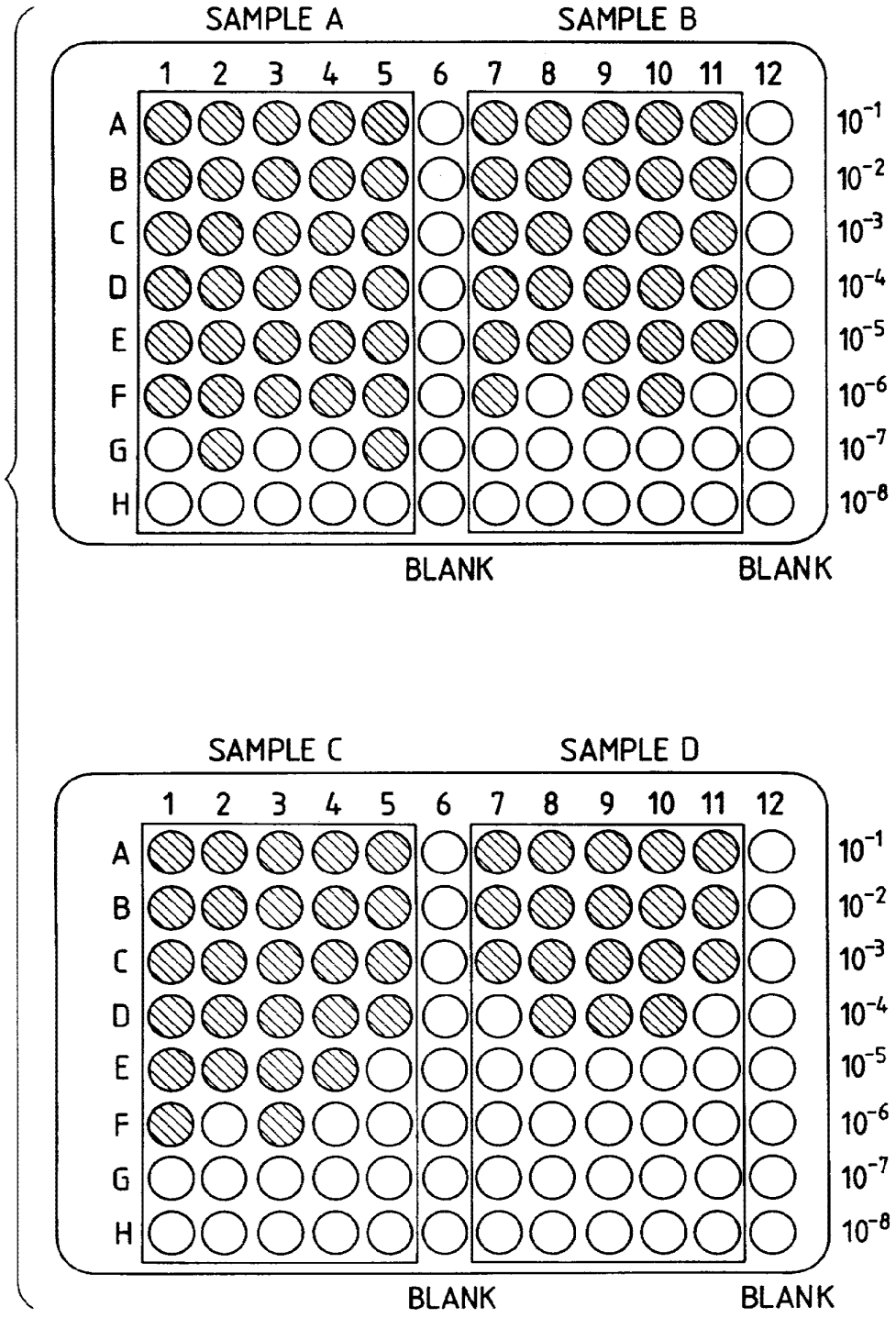

SAMPLE A
SAMPLE B
1 2 3 4 5 6 7 8 9 10 11 12
A
B
C
D
E
F
G
H
10^{-1}
10^{-2}
10^{-3}
10^{-4}
10^{-5}
10^{-6}
10^{-7}
10^{-8}
BLANK
BLANK
SAMPLE C
SAMPLE D
1 2 3 4 5 6 7 8 9 10 11 12
A
B
C
D
E
F
G
H
10^{-1}
10^{-2}
10^{-3}
10^{-4}
10^{-5}
10^{-6}
10^{-7}
10^{-8}
BLANK
BLANK

FIG. 5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SAMPLE B
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A
B
C
D
E
F
G
H
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10^{-2}
10^{-3}
10^{-4}
10^{-5}
10^{-6}
10^{-7}
10^{-8}
BLANK
BLANK
SAMPLE C
SAMPLE D
1 2 3 4 5 6 7 8 9 10 11 12
A
B
C
D
E
F
G
H
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10^{-5}
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10^{-7}
10^{-8}
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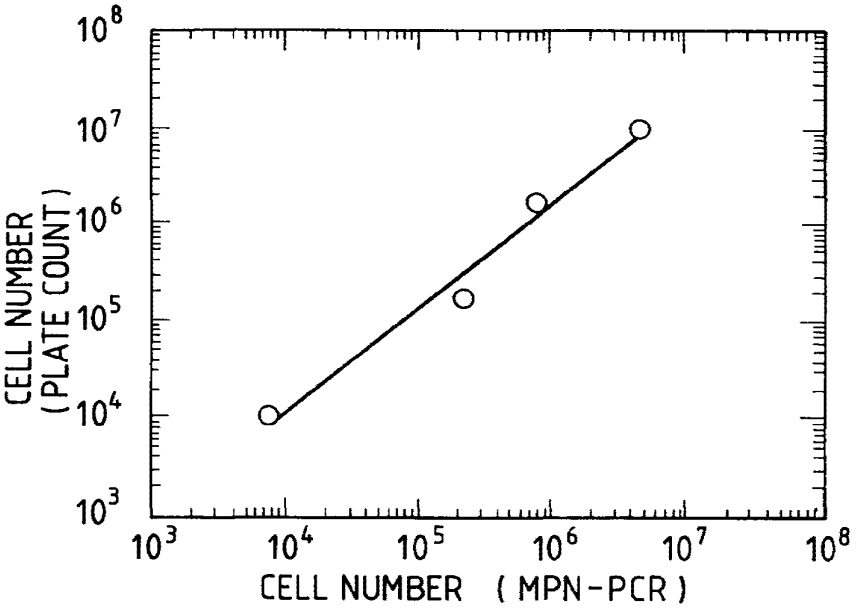
BLANK

FIG. 6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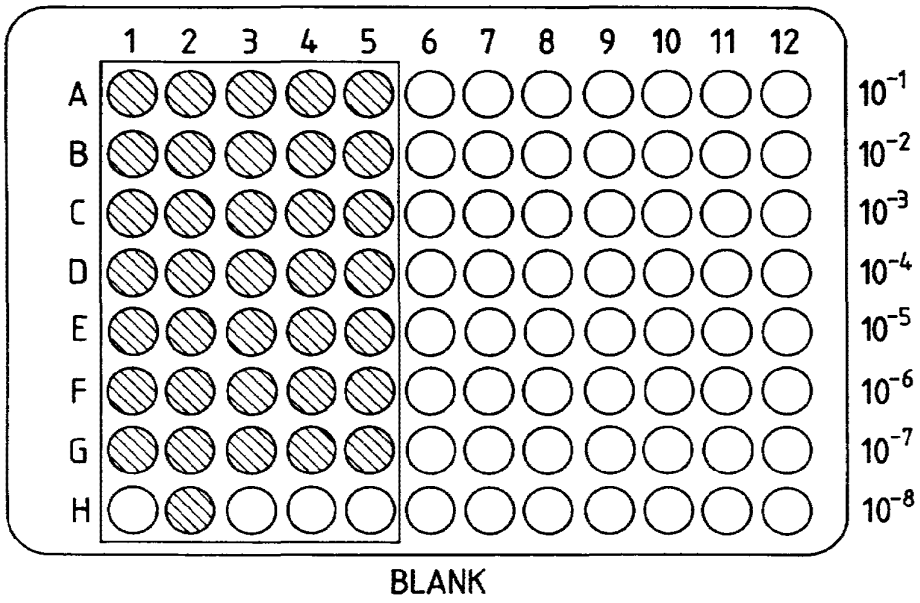
10^8
10^7
10^6
10^5
10^4
10^3
10^3 10^4 10^5 10^6 10^7 10^8
CELL NUMBER (MPN-PCR)

FIG. 7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B 10^{-2}
C 10^{-3}
D 10^{-4}
E 10^{-5}
F 10^{-6}
G 10^{-7}
H 10^{-8}
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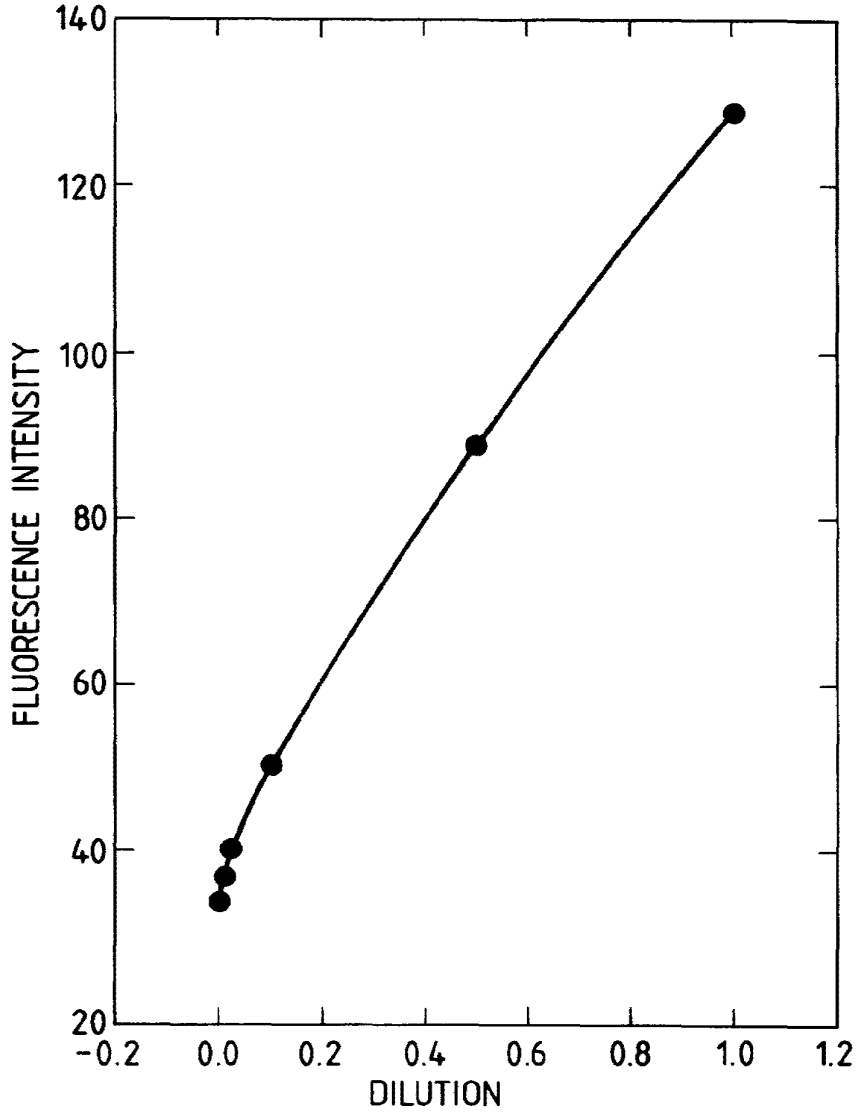
FIG. 8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120
100
80
60
40
20
FLUORESCENCE INTENSITY
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0.0
0.2
0.4
0.6
0.8
1.0
1.2
DILUTION

FIG. 9A

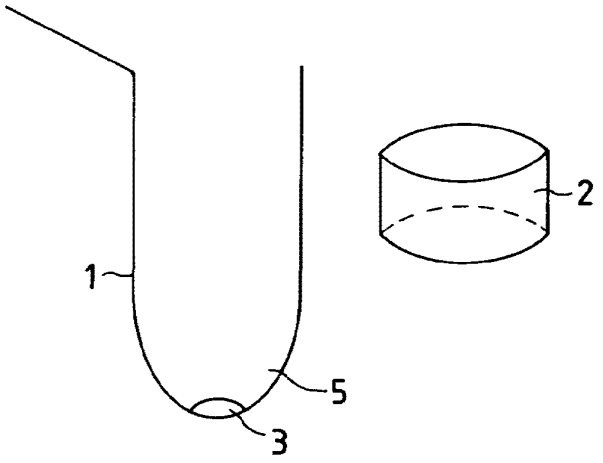


FIG. 9B

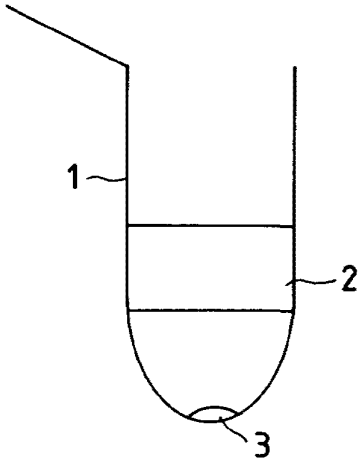
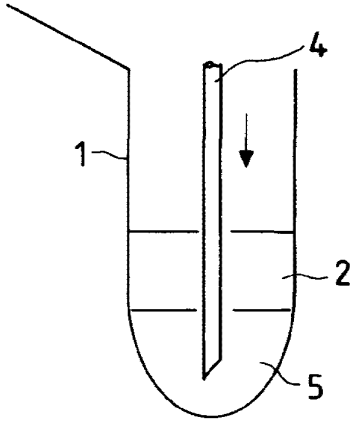


FIG. 9C



European Patent Office

EUROPEAN SEARCH REPORT

Application Number
EP 94 10 9568

DOCUMENTS CONSIDERED TO BE RELEVANT

Category	Citation of document with indication, where appropriate, of relevant passages	Relevant to claim	CLASSIFICATION OF THE APPLICATION (Int.Cl.6)
X	EP-A-0 487 218 (TOSOH CORPORATION) 27 May 1992 * the whole document *	1	C12Q1/68 C09B57/00 G01N33/52
X	EP-A-0 512 334 (F. HOFFMANN- LA ROCHE AG) 11 November 1992 * the whole document *	1	
A	CYTOMETRY, vol.5, no.4, July 1984, NEW YORK pages 339 - 347 S. A. LATT ET AL. 'New Fluorochromes, Compatible With High Wavelength Excitation, for Flow Cytometric Analysis of Cellular Nucleic Acids.'		
A	US-A-4 555 396 (D. S. FRANK ET AL.)		
A	BER. BUNSENGES. PHYS. CHEM., vol.96, no.7, 1992 pages 880 - 886 G. HAUCKE ET AL 'Absorption and fluorescence of Pyrylium Salts.'		TECHNICAL FIELDS SEARCHED (Int.Cl.6) G01N C09B C12Q
A	APPLIED PHYSICS, vol.3, 1974 pages 81 - 88 D. BASTING ET AL. 'New Laser Dyes'		

The present search report has been drawn up for all claims

Place of search	Date of completion of the search	Examiner
THE HAGUE	20 September 1994	Cartagena y Abella,P

CATEGORY OF CITED DOCUMENTS

X : particularly relevant if taken alone
Y : particularly relevant if combined with another document of the same category
A : technological background
O : non-written disclosure
P : intermediate document

T : theory or principle underlying the invention
E : earlier patent document, but published on, or after the filing date
D : document cited in the application
L : document cited for other reasons

& : member of the same patent family, corresponding document

EPO FORM 1503 03.82 (P04C01)

Electronic Patent Application Fee Transmittal

Application Number:	13071105
Filing Date:	24-Mar-2011
Title of Invention:	Digital Amplification
First Named Inventor/Applicant Name:	Bert VOGELSTEIN
Filer:	Sarah Anne Kagan./Jennifer Brady
Attorney Docket Number:	001107.00866

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:	16304565
Application Number:	13071105
International Application Number:	
Confirmation Number:	3361
Title of Invention:	Digital Amplification
First Named Inventor/Applicant Name:	Bert VOGELSTEIN
Customer Number:	11332
Filer:	Sarah Anne Kagan./Jennifer Brady
Filer Authorized By:	Sarah Anne Kagan.
Attorney Docket Number:	001107.00866
Receipt Date:	12-JUL-2013
Filing Date:	24-MAR-2011
Time Stamp:	16:13:03
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	3178
Deposit Account	190733
Authorized User	

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

Charge any Additional Fees required under 37 C.F.R. Section 1.16 (National application filing, search, and examination fees)

Charge any Additional Fees required under 37 C.F.R. Section 1.17 (Patent application and reexamination processing fees)

Charge any Additional Fees required under 37 C.F.R. Section 1.19 (Document supply fees)

Charge any Additional Fees required under 37 C.F.R. Section 1.20 (Post Issuance fees)

Charge any Additional Fees required under 37 C.F.R. Section 1.21 (Miscellaneous fees and charges)

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)	IDS_off_CIPO_OA_dtd_04-12-2013.PDF	612410 d8c62ba4bb7a03cfe9cbf4399ff3d100aaf7bf9	no	4
Warnings:					
Information:					
A U.S. Patent Number Citation or a U.S. Publication Number Citation is required in the Information Disclosure Statement (IDS) form for autoloading of data into USPTO systems. You may remove the form to add the required data in order to correct the Informational Message if you are citing U.S. References. If you chose not to include U.S. References, the image of the form will be processed and be made available within the Image File Wrapper (IFW) system. However, no data will be extracted from this form. Any additional data such as Foreign Patent Documents or Non Patent Literature will be manually reviewed and keyed into USPTO systems.					
2	Non Patent Literature	1NPL_CIPO_OA_04-12-2013.PDF	33886 dccc396c7f0b32dfc083dad0a8838a900ea40a1f	no	2
Warnings:					
The page size in the PDF is too large. The pages should be 8.5 x 11 or A4. If this PDF is submitted, the pages will be resized upon entry into the Image File Wrapper and may affect subsequent processing					
Information:					
3	Non Patent Literature	PIATEK_etal_NatureBiotechnology.PDF	494305 24951f50f0ed0adfcf0f813363a7022ee14859e0	no	5
Warnings:					
Information:					
4	Foreign Reference	EP0643140A1_Yamamoto.PDF	3299269 f9008272d78be22a1056bccf3a65bc0022671043	no	81
Warnings:					
Information:					
5	Fee Worksheet (SB06)	fee-info.pdf	30158 86860c841e4932fc6a3f98d785dc081a7e2fe58f	no	2
Warnings:					
Information:					
		Total Files Size (in bytes):	4470028		

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.

PATENT

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re Application of	)	
	)	
Bert VOGELSTEIN et al.	)	Examiner: WOOLWINE, Samuel C.
	)	
Serial No. 13/071,105	)	
	)	Group Art Unit: 1637
	)	
Filed: March 24, 2011	)	Confirmation No. 3361
	)	
For: **DIGITAL AMPLIFICATION**	)	Atty. Dkt. No. 001107.00866

RESPONSE TO OFFICE ACTION

Commissioner of Patents
P.O. Box 1450
Alexandria, VA 22313-1450

Sir:

In response to the office action mailed June 27, 2013, applicants request entry of the amendment and reconsideration of the patentability of the claims in view of the remarks.

A request for consideration under the AFCPP 2.0 accompanies this paper. No petition for extension of time accompanies this submission. The Commissioner is authorized to charge any fees which may be required or credit any overpayment to our Deposit Account 19-0733.

IN THE CLAIMS

Please replace the following claim set for that currently of record.

1. -48. (Cancelled)

49. (Currently amended) A method for detecting quantity of a genetic sequence in a mixed population of human genomic nucleic acid sequences comprising at least a first and a second human genomic sequence, wherein the first sequence is a wild-type sequence of an allele and a second sequence is a mutant sequence of the allele, comprising:

distributing or diluting a mixed population of cell-free, human genomic nucleic acid template molecules from a sample in which the fraction of mutant alleles is less than 20 %, into a set comprising at least ~~ten~~ fifteen assay samples such that said at least ~~ten~~ fifteen assay samples each comprises less than ten template molecules;

amplifying the template molecules in the assay samples, wherein an assay sample with a single template molecule forms homogeneous amplification products in the assay sample;

analyzing by determining nucleic acid sequence of amplification products in the assay samples of the set with homogeneous amplification products to determine a first number of assay samples in the set which contain the first sequence and a second number of assay samples in the set which contain the second sequence;

comparing the first number to the second number to ascertain a ratio which reflects the composition of the mixed population;

identifying a mutation in the mixed population if a statistically significant fraction of assay samples comprises the second sequence.

50. (Previously Presented) The method of claim 49 wherein the assay samples of the set have on average 0.5 molecules of template.

51. (Previously Presented) The method of claim 49 wherein between 0.1 and 0.9 of the assay samples yield an amplification product.

52. (Previously Presented) The method of claim 49 wherein the mixed population of nucleic acid sequences is distributed or diluted to a single template molecule level in the assay samples.

53. (Currently amended) The method of claim 49 wherein the mixed population of nucleic acid sequences is from a ~~tissue or~~ body sample.

54. (Currently amended) The method of claim ~~49~~ 53 wherein the mixed population of nucleic acids sequences is from a body sample selected from the group consisting of stool, blood, and lymph nodes.

55. (Cancelled)

56. (Previously Presented) The method of claim 49 wherein the mixed population of nucleic acids sequences is distributed or diluted such that at least twenty assay samples comprise less than ten template molecules.

57. (Previously Presented) The method of claim 49 wherein the mixed population of nucleic acids sequences is distributed or diluted such that at least twenty-five assay samples comprise less than ten template molecules.

58. (Previously Presented) The method of claim 49 wherein the mixed population of nucleic acids sequences is distributed or diluted such that at least thirty assay samples comprise less than ten template molecules.

59. (Previously Presented) The method of claim 49 wherein the mixed population of nucleic acids sequences is distributed or diluted such that at least forty assay samples comprise less than ten template molecules.

60. (Previously Presented) The method of claim 49 wherein the mixed population of nucleic acids sequences is distributed or diluted such that at least fifty assay samples comprise less than ten template molecules.

61. (Previously Presented) The method of claim 49 wherein the mixed population of nucleic acids sequences is distributed or diluted such that at least seventy-five assay samples comprise less than ten template molecules.

62. (Previously Presented) The method of claim 49 wherein the mixed population of nucleic acids sequences is distributed or diluted such that at least one hundred assay samples comprise less than ten template molecules.

63. (Previously Presented) The method of claim 49 wherein the mixed population of nucleic acids sequences is distributed or diluted such that at least five hundred assay samples comprise less than ten template molecules.

64. (Previously Presented) The method of claim 49 wherein the mixed population of nucleic acids sequences is distributed or diluted such that at least one thousand assay samples comprise less than ten template molecules.

65. (Currently amended) The method of claim 49 wherein the mixed population of nucleic acids sequences is distributed or diluted such that at least one thousand assay samples are distributed or diluted to a single template molecule level.

66. (Previously Presented) The method of claim 49 wherein the mixed population of nucleic acids sequences is distributed or diluted such that at least one thousand assay samples has on average 0.5 molecules of template.

67. (Previously Presented) The method of claim 49 wherein the mixed population of nucleic acids sequences is distributed or diluted such that between 0.1 and 0.9 of at least one thousand assay samples yield an amplification product.

68. (Previously Presented) The method of claim 49 wherein the mixed population of nucleic acids sequences is distributed or diluted such that one half of at least one thousand assay samples have one template molecule.

69. (New) The method of claim 49 wherein the mutation is a somatic mutation.

70. (New) The method of claim 49 wherein the mutation is a cancer gene mutation.

71. (New) The method of claim 49 wherein the template molecules are from a population of cells which are not purely tumor cells.

72. (New) The method of claim 49 wherein between 1% and 10 % of the alleles in said human genomic nucleic acid template molecules are the mutant sequence of the allele.

73. (New) The method of claim 49 wherein the mixed population of nucleic acid sequences is from a tissue.

74. (New) A method for detecting quantity of a genetic sequence in a mixed population of human genomic nucleic acid sequences comprising at least a first and a second human genomic sequence, wherein the first sequence is a wild-type sequence of an allele and a second sequence is a mutant sequence of the allele, comprising:

distributing or diluting a mixed population of cell-free, human genomic nucleic acid template molecules into a set comprising at least fifteen assay samples such that said at least fifteen assay samples comprises an average of 0.5 molecules of template.;

amplifying the template molecules in the assay samples, wherein an assay sample with a single template molecule forms homogeneous amplification products in the assay sample;

analyzing by determining nucleic acid sequence of amplification products in the assay samples of the set with homogeneous amplification products to determine a first number of assay samples in the set which contain the first sequence and a second number of assay samples in the set which contain the second sequence;

comparing the first number to the second number to ascertain a ratio which reflects the composition of the mixed population;

identifying a mutation in the mixed population if a statistically significant fraction of assay samples comprises the second sequence.

.

IN THE SPECIFICATION

Please substitute at page 3, last paragraph, and first paragraph on page 4, with the following:

According to another embodiment of the invention, a molecular beacon probe is provided. It comprises an oligonucleotide with a stem-loop structure having a photoluminescent dye at one of the 5' or 3' ends and a quenching agent at the opposite 5' or 3' end. The loop consists of 16 base pairs which has a T_m of 50-51 ~~≡~~ °C. The stem consists of 4 base pairs having a sequence 5'-CACG-3'.

A second type of molecular beacon probe is provided in another embodiment. It comprises an oligonucleotide with a stem-loop structure having a photoluminescent dye at one of the 5' or 3' ends and a quenching agent at the opposite 5' or 3' end. The loop consists of 19-20 base pairs and has a T_m of 54-56 ~~≡~~ °C. The stem consists of 4 base pairs having a sequence 5'-CACG-3'.

Please substitute the paragraph spanning pages 7 and 8, with the following:

Digital amplification can be used to detect mutations present at relatively low levels in the samples to be analyzed. The limit of detection is defined by the number of wells that can be analyzed and the intrinsic mutation rate of the polymerase used for amplification. 384 well PCR plates are commercially available and 1536 well plates are on the horizon, theoretically allowing sensitivities for mutation detection at the .about.0.1% level. It is also possible that Digital Amplification can be performed in microarray format, potentially increasing the sensitivity by another order of magnitude. This sensitivity may ultimately be limited by polymerase errors. The effective error rate in PCR as performed under our conditions was 1.1%, i.e., four out of 351 PCR products derived from WT DNA sequence appeared to contain a mutation by RED/GREEN ratio criteria. However, any individual mutation (such as a G to T transversion at the second position of codon 12 of c-Ki-Ras), ~~are~~ is expected to occur in <1 in 50 of these polymerase-

generated mutants (there are at least 50 base substitutions within or surrounding codons 12 and 13 that should yield high RED/GREEN ratios). Determining the sequence of the putative mutants in the positive wells, by direct sequencing as performed here or by any of the other techniques, provides unequivocal validation of a prospective mutation: a significant fraction of the mutations found in individual wells should be identical if the mutation occurred in vivo. Significance can be established through rigorous statistical analysis, as positive signals should be distributed according to Poisson probabilities. Moreover, the error rate in particular Digital Amplification experiments can be precisely determined through performance of Digital Amplification on DNA templates from normal cells.

Remarks

The amendments to claim 49 are fully supported and do not add new matter. The recitation of fifteen assay samples was formerly recited in claim 55. The recitation of fraction of mutant alleles is supported in the specification at page 2, lines 5-7.

Claim 53 is amended to separate alternative recitations. Claim 54 is amended to clarify its intended meaning.

Claim 55 are cancelled in view of the amendment to independent claim 49 which incorporates its recitation.

Claim 65 is amended for internal consistency of its recitations.

New claims 69-73 are fully supported in the application as originally filed. Claim 69 is supported at page 1, line 12, and page 2, line 15. Claim 70 is supported at page 9, in the table at row 2, column 2. Claim 71 is supported at page 10, last full sentence. Claim 72 is supported at page 21, last paragraph. Claim 73 is supported by claim 54 prior to its amendment.

Claim 74 is supported by claim 49, prior to its amendment and claim 50.

The rejection of claims 49 and 51-53 under § 103(a)

Claims 49 and 51-53 stand rejected as unpatentable over Ruano (PNAS 87:6296-6300, 1990). Ruano is cited as having met the limitations of the first three steps of (a) distributing or diluting, (b) amplifying, and (c) analyzing. The last two steps of (d) comparing and (e) identifying were asserted as not taught, but as just being obvious. The Patent and Trademark Office's construction of the claim terms "wild- type" and "mutant" as encompassing two different SNPs was a necessary part of its rejection. While applicant does not concur in the construction of these terms, it has added further terms to independent claim 49 to clarify the context and scope of the claimed method.

Claim 49 as amended recites that the fraction of mutant alleles in the sample is less than 20%. This is a limitation that distinguishes over Ruano's mere SNPs, one on each of two homologous chromosomes, present in a 1:1 ratio; the mutant alleles of the present invention are present in a minority of nucleic acids in a sample. Claim 49 as amended also recites a minimum of 15 assay samples that are amplified and analyzed and compared. Ruano did not teach more than 10 samples, and so did not teach the number of samples now recited in claim 49. See office action at page 6, lines 12-13.

Ruano's determination of a haplotype does not teach or suggest a mutant allele which is present at less than 20% in the sample. SNPs, as taught by Ruano, are typically present at a fraction of ~50%.

Finally, there would be no reason to assay fifteen assay samples for Ruano's method. If two alleles are present in a 1:1 ratio in a sample, then ten assay samples are more than sufficient to achieve excellent detection, as Ruano demonstrated.

The obtaining of a haplotype, as Ruano taught, is conceptually distinct and does not suggest detection of rare alleles in a mixed population present at <20%.

The method of independent claim 49 as amended is not obvious over Ruano. For at least these reasons dependent claims 51, 52, and 53 are also not obvious.

Please withdraw this rejection.

Rejection of claims 50 and 55-68 under § 103(a)

Claims 50 and 55-68 stand rejected as obvious over Ruano (PNAS 87:6296-6300, 1990) in view of Stephens (Am. J. Hum. Gen 46:1149-1155 , 1990). Claim 55 is cancelled.

Claim 50 recites (as do claims 66 and 74) that the assay samples have on average 0.5 molecules of the template. Claims 56-68 recite at least 20 and up to at least 1000 assay samples. The Patent and Trademark Office acknowledges that Ruano does not teach either of these parameters. However, Stephens is cited to remedy the deficiency of Ruano.

Stephens is cited as teaching in Table 1 the probability of success at having one or more vials having one but not both haplotypes in it. Stephens teaches that for 10 vials and 1 haploid equivalent in a vial, as used by Ruano, one has a 0.9985 chance of success. In fact, of the choices shown in Table 1, it appears that Ruano used the optimal conditions. Contrary to the assertion of the Patent and Trademark Office, Table 1 would not have motivated one of ordinary skill in the art to use even more assay vials. A 0.9985 chance of success would be considered to be quite high and increasing the number of vials would likely yield a very small increase. Stephens, contrary to the Patent and Trademark Office's assertion, would lead one of skill in the art to do exactly what Ruano had done—10 samples and single molecule dilution. No modification of the method of Ruano would have been suggested.

Similarly, contrary to the assertion of the Patent and Trademark Office, Stephens would not motivate one of skill in the art to modify Ruano's teaching of single molecule dilution to use

0.5 molecules, as recited in claim 50. Inspection of Stephens' Table 1 would lead one of skill in the art to realize that modifying Ruano's method in that way would lead to a diminished success rate.

One of ordinary skill in the art would not have found Stephens' teaching to be motivational for any empirical optimization. Stephens explicitly taught the theoretical underpinnings for the parameters that Ruano chose—they were the optimum for the method that Ruano was performing. Once optimized, those of skill in the art are not likely to seek variations.

Moreover, for at least the same reasons as for independent claim 49, dependent claims 50 and 56-68 are not obvious over the combination of Ruano and Stephens. Stephens does not address any of the deficiencies of Ruano with regard to claim 49 as amended.

Please withdraw this rejection.

Rejection of claims 55-68 under § 103 (a)

Claims 55-68 stand rejected over Ruano (PNAS 87:6296-6300, 1990), as applied above, in view of Kruglyak (Nature Genetics 22:139-144, 1999). Claim 55 is cancelled.

As acknowledged by the Patent and Trademark Office, Ruano did not teach more than 10 assay samples. Use of more than 10 assay samples is recited in each of claims 56-68.

Kruglyak is cited as suggesting haplotype analysis at many different SNPs. This, the Patent and Trademark Office asserts, would have made it obvious to use the method of Ruano for haplotype analysis at many different sites throughout the genome. The rejection concludes that this would have necessitated the use of up to 1000 or even more "single molecule" samples. Page 8, lines 3-4.

What the Patent and Trademark Office proposes is that a sample would be distributed into 1000 or more assays, which might have, *arguendo,* fulfilled the limitations of the step of "distributing or diluting." It appears that the hypothesized experiment would determine different SNPs in different subsets of the 1000 assay samples. As discussed above, optimized results for the Ruano type haplotype assay for one SNP (allele) can be determined with just 10 assay samples. Thus it appears that many subsets of 10 assay samples would be used to determine different SNPs. The hypothesized experiment would not fulfill the recitations of the step of "analyzing by determining nucleic acid sequence." That step requires that the number of assay samples with the first sequence (wild type allele) and the number of assay samples with the

second sequence (mutant allele) be determined in the assay samples of the set. The set is defined with a minimum of at least 20, 25, 30, 40, 50, 75, 100, 500, or 1000 assay samples in claims 56-68. The hypothesized experiment would not, therefore, fulfill all elements of the claims.

Moreover, for at least the same reasons as for independent claim 49, dependent claims 56-68 are not obvious over the combination of Ruano and Kruglyak. Kruglyak does not address any of the deficiencies of Ruano with regard to independent claim 49 as amended.

Please withdraw this rejection.

The rejection of claim 54 under § 103(a)

Dependent claim 54 stands rejected as unpatentable over Ruano (PNAS 87:6296-6300, 1990) in view of Kulozik. (Am J. Hum Gen 39:239-244, 1986). Kulozik is cited as teaching blood as a source of genomic DNA for haplotyping. Kulozik does not remedy the deficiencies of Ruano with regard to independent claim 49. Kulozik teaching nothing about assaying for alleles present at less than 20% using at least 15 assay samples.

For at least the same reasons as claim 49, claim 54 is patentable over Ruano in view of Kulozik. Please withdraw this rejection.

Respectfully submitted,

Date: *25 September, 2013*

By: */Sarah A. Kagan/*
Sarah A. Kagan
Registration No. 32,141

Banner & Witcoff, Ltd.
Customer No. 11332

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.:	Application No.:	Filing Date:
001107.00866	13/071,105	March 24, 2011
First Named Inventor:	**Title:**	
VOGELSTEIN, Bert	DIGITAL AMPLIFICATION	

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	Date
/Sarah A. Kagan/	25 September 2013
Name (Print/Typed) Sarah A. Kagan	**Practitioner Registration No.** 32141

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 _____ forms are submitted.

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.

Electronic Acknowledgement Receipt

EFS ID:	16956633
Application Number:	13071105
International Application Number:	
Confirmation Number:	3361
Title of Invention:	Digital Amplification
First Named Inventor/Applicant Name:	Bert VOGELSTEIN
Customer Number:	11332
Filer:	Sarah Anne Kagan.
Filer Authorized By:	
Attorney Docket Number:	001107.00866
Receipt Date:	25-SEP-2013
Filing Date:	24-MAR-2011
Time Stamp:	17:05:08
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	Response After Final Action	00866amend.pdf	129272 b7ccdfc2a39745ad49db95b1d84a5e436bf3cb52	no	11

Warnings:	
Information:	

2	After Final Consideration Program Request	00866afcpp.pdf	203584 d6f5b3245f86f6572786463d973749d17838f3dd	no	2

Warnings:	
Information:	
Total Files Size (in bytes):	332856

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
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 13/071,105	Filing Date 03/24/2011	☐ 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 **09/25/2013**

	(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))	* 25	Minus	** 68	= 0	X $80 =	0
Independent (37 CFR 1.16(h))	* 2	Minus	***6	= 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
/GOIGA DUCKETT/

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.
13/071,105	03/24/2011	Bert VOGELSTEIN	001107.00866	3361

11332 7590 10/07/2013

Banner & Witcoff, Ltd.
Attorneys for client 001107
1100 13th Street N.W.
Suite 1200
Washington, DC 20005-4051

EXAMINER	
WOOLWINE, SAMUEL C	
ART UNIT	PAPER NUMBER
1637	

MAIL DATE	DELIVERY MODE
10/07/2013	PAPER

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.

Advisory Action Before the Filing of an Appeal Brief	**Application No.** 13/071,105	**Applicant(s)** VOGELSTEIN ET AL.	
	Examiner SAMUEL WOOLWINE	**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 25 September 2013 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 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: _____.

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: 49-68.
Claim(s) withdrawn from consideration: .

/Samuel Woolwine/ Primary Examiner	

Continuation of 11. does NOT place the application in condition for allowance because: Applicant's request for entry into AFCP 2.0 is acknowledged, but is denied because the response cannot be reviewed and a search conducted in the limited amount of time authorized for this pilot program. Therefore, the response is being reviewed under pre-pilot practice. The amendment to claim 49 would require further search and consideration since in the art of record, Ruano is investigating a genetic locus wherein, in the sample, it was determined that the fraction of each allele was 50%. Therefore, further search and consideration would be required, which the Examiner cannot complete in the alloted time under AFCP 2.0. Since the amendment is not entered, the rejections of record are maintained.

PATENT

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re Application of	)	
	)	
Bert VOGELSTEIN et al.	)	Examiner: WOOLWINE, Samuel C.
	)	
Serial No. 13/071,105	)	
	)	Group Art Unit: 1637
	)	
Filed: March 24, 2011	)	Confirmation No. 3361
	)	
For: **DIGITAL AMPLIFICATION**	)	Atty. Dkt. No. 001107.00866

RESPONSE TO OFFICE ACTION

Commissioner of Patents
P.O. Box 1450
Alexandria, VA 22313-1450

Sir:

In response to the office action mailed June 27, 2013, applicants request entry of the amendment and reconsideration of the patentability of the claims in view of the remarks.

A request for consideration under the AFCPP 2.0 accompanies this paper. No petition for extension of time accompanies this submission. The Commissioner is authorized to charge any fees which may be required or credit any overpayment to our Deposit Account 19-0733.

Do not enter
/SW/

PTO/SB/30EFS (07-09)
Approved for use through 07/31/2012. OMB 0651-0031
U.S. Patent and Trademark Office; U.S. DEPARTMENT OF COMMERCE

REQUEST FOR CONTINUED EXAMINATION(RCE)TRANSMITTAL
(Submitted Only via EFS-Web)

Application Number	13/071,105	Filing Date	2011-03-24	Docket Number (if applicable)	001107.00866	Art Unit	1637
First Named Inventor	Vogelstein et al.			Examiner Name	Samuel C. Woolwine		

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. The Instruction Sheet for this form is located at WWW.USPTO.GOV

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).

☒ 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.

☐ Consider the arguments in the Appeal Brief or Reply Brief previously filed on _____

☐ Other _____

☐ Enclosed

☐ Amendment/Reply

☐ Information Disclosure Statement (IDS)

☐ Affidavit(s)/ Declaration(s)

☐ Other _____

MISCELLANEOUS

☐ Suspension of action on the above-identified application is requested under 37 CFR 1.103(c) for a period of months _____ (Period of suspension shall not exceed 3 months; Fee under 37 CFR 1.17(i) required)

☐ Other _____

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 any underpayment of fees, or credit any overpayments, to Deposit Account No 190733

SIGNATURE OF APPLICANT, ATTORNEY, OR AGENT REQUIRED

☒ Patent Practitioner Signature

☐ Applicant Signature

PTO/SB/30EFS (07-09)
Approved for use through 07/31/2012. OMB 0651-0031
U.S. Patent and Trademark Office; U.S. DEPARTMENT OF COMMERCE

Signature of Registered U.S. Patent Practitioner			
Signature	/Sarah A. Kagan/	Date (YYYY-MM-DD)	2013-10-14
Name	Sarah A. Kagan	Registration Number	32141

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.

If you need assistance in completing the form, call 1-800-PTO-9199 and select option 2.

Privacy Act Statement

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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.

PTO/SB/22 (09-11)
Approved for use through 07/31/2012. OMB 0651-0031
U.S. Patent and Trademark Office; U.S. DEPARMENT OF COMMERCE

PETITION FOR EXTENSION OF TIME UNDER 37 CFR 1.136(a)	Docket Number (Optional) 001107.00866
Application Number 13/071,105	Filed March 24, 2011
For Digital Amplification	
Art Unit 1637	Examiner Samuel C. Woolwine

This is a request under the provisions of 37 CFR 1.136(a) to extend the period for filing a reply in the above identified application.

The requested extension and fee are as follows (check time period desired and enter the appropriate fee below):

	Fee	Small Entity Fee	
☑ One month (37 CFR 1.17(a)(1))	$150	$75	$ 200.00
☐ Two months (37 CFR 1.17(a)(2))	$560	$280	$
☐ Three months (37 CFR 1.17(a)(3))	$1270	$635	$
☐ Four months (37 CFR 1.17(a)(4))	$1980	$990	$
☐ Five months (37 CFR 1.17(a)(5))	$2690	$1345	$

☐ Applicant claims small entity status. See 37 CFR 1.27.

☐ A check in the amount of the fee is enclosed.

☐ Payment by credit card. Form PTO-2038 is attached.

☐ The Director has already been authorized to charge fees in this application to a Deposit Account.

☑ The Director is hereby authorized to charge any fees which may be required, or credit any overpayment, to Deposit Account Number 190733.

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.

I am the

☐ applicant/inventor.

☐ assignee of record of the entire interest. See 37 CFR 3.71.
Statement under 37 CFR 3.73(b) is enclosed (Form PTO/SB/96).

☑ attorney or agent of record. Registration Number 32,141

☐ attorney or agent under 37 CFR 1.34.
Registration number if acting under 37 CFR 1.34 _____

/Sarah A. Kagan/ — Signature

14 October 2013 — Date

Sarah A. Kagan — Typed or printed name

(202) 824-3000 — Telephone Number

NOTE: Signatures of all the inventors or assignees of record of the entire interest or their representative(s) are required. Submit multiple forms if more than one signature is required, see below.

☑ Total of 1 forms are submitted.

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Electronic Patent Application Fee Transmittal

Application Number:	13071105
Filing Date:	24-Mar-2011
Title of Invention:	Digital Amplification
First Named Inventor/Applicant Name:	Bert VOGELSTEIN
Filer:	Sarah Anne Kagan./Jennifer Hazzard
Attorney Docket Number:	001107.00866

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:				
Extension - 1 month with $0 paid	1251	1	200	200

Description	Fee Code	Quantity	Amount	Sub-Total in USD($)
Miscellaneous:				
Request for Continued Examination	1801	1	1200	1200
		Total in USD ($)		**1400**

Electronic Acknowledgement Receipt

EFS ID:	17118926
Application Number:	13071105
International Application Number:	
Confirmation Number:	3361
Title of Invention:	Digital Amplification
First Named Inventor/Applicant Name:	Bert VOGELSTEIN
Customer Number:	11332
Filer:	Sarah Anne Kagan./Jennifer Hazzard
Filer Authorized By:	Sarah Anne Kagan.
Attorney Docket Number:	001107.00866
Receipt Date:	14-OCT-2013
Filing Date:	24-MAR-2011
Time Stamp:	15:35:33
Application Type:	Utility under 35 USC 111(a)

Payment information:

Submitted with Payment	yes
Payment Type	Deposit Account
Payment was successfully received in RAM	$1400
RAM confirmation Number	12721
Deposit Account	190733
Authorized User	

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

Charge any Additional Fees required under 37 C.F.R. Section 1.16 (National application filing, search, and examination fees)

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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)	RCE.PDF	697782 57ce7fbcb091e37460bf892be8794128d0f1388f	no	3
Warnings:					
Information:					
2	Extension of Time	Petition-for-EOT.PDF	289733 ab98e0c088a30d3e5e7a0e66d2f5371be7e6d03f	no	2
Warnings:					
Information:					
3	Fee Worksheet (SB06)	fee-info.pdf	32235 4ac8a4b2e398284f2ddcf6117b375525ddd59828	no	2
Warnings:					
Information:					
		Total Files Size (in bytes):	1019750		

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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.

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 13/071,105	Filing Date 03/24/2011	☐ 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 **10/14/2013**

	(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))	* 25	Minus	** 68	= 0	X $80 =	0
Independent (37 CFR 1.16(h))	* 2	Minus	***6	= 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
/LINDA HUMES/

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APPLICATION NO.	FILING DATE	FIRST NAMED INVENTOR	ATTORNEY DOCKET NO.	CONFIRMATION NO.
13/071,105	03/24/2011	Bert VOGELSTEIN	001107.00866	3361

11332 7590 12/30/2013

Banner & Witcoff, Ltd.
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EXAMINER	
WOOLWINE, SAMUEL C	
ART UNIT	PAPER NUMBER
1637	

MAIL DATE	DELIVERY MODE
12/30/2013	PAPER

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.

Office Action Summary	Application No. 13/071,105	Applicant(s) VOGELSTEIN ET AL.	
	Examiner SAMUEL WOOLWINE	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 *10/14/2013*.

☐ 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) *49-54 and 56-74* is/are pending in the application.

5a) Of the above claim(s) _____ is/are withdrawn from consideration.

6)☒ Claim(s) *49-54 and 56-73* is/are allowed.

7)☒ Claim(s) *74* is/are rejected.

8)☒ Claim(s) *74* 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 *07/12/2013*.

3) ☐ Interview Summary (PTO-413) Paper No(s)/Mail Date. _____ .

4) ☐ Other: _____.

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

DETAILED ACTION

Continued Examination Under 37 CFR 1.114

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 09/25/2013 has been entered.

With regard to the Office action mailed 06/27/2013, all rejections are withdrawn in view of the amendments to the claims. However, the rejection under 35 USC 103 previously applied to claim 50 has been applied to new claim 74 as set forth below.

Claim Objections

Claim 74 is objected to because of the following informalities: there is an inadvertent period at the end of line 7, just before the semi-colon. Appropriate correction is required.

Claim Rejections - 35 USC § 103

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.

The factual inquiries set forth in *Graham v. John Deere Co.*, 383 U.S. 1, 148 USPQ 459 (1966), that are applied for establishing a background for determining obviousness under pre-AIA 35 U.S.C. 103(a) are summarized as follows:

1. Determining the scope and contents of the prior art.

2. Ascertaining the differences between the prior art and the claims at issue.

3. Resolving the level of ordinary skill in the pertinent art.

4. Considering objective evidence present in the application indicating obviousness or nonobviousness.

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).

Claim 74 is rejected under 35 U.S.C. 103(a) as being unpatentable over Ruano et al (PNAS 87:6296-6300 (1990), cited on the IDS of 10/10/2012) in view of Stephens et al (Am J Hum Gen 46:1149-1155 (1990), cited on the IDS of 10/10/2012).

With regard to claim 74, Ruano taught a method comprising:

distributing or diluting a mixed population of cell-free, human genomic nucleic acid template molecules into a set comprising at least ten assay samples such that said at least 10 assay samples each comprises less than ten template molecules

See figure 3a; Ruano diluted and distributed genomic DNA into ten samples (lanes 4-13) having 1 template copy.

amplifying the template molecules in the assay samples, wherein an assay sample with a single template molecule forms homogeneous amplification products in the assay sample

See figure 3a; “booster PCR. In addition, amplification of a single template molecule would inherently form a homogeneous product. Note in the caption of figure 3, Ruano states: "...while product amplified from 1000 template copies contains both alleles (lane 1), SMD products from this series contain one or the other." SMD stands for "single molecule dilution".

analyzing by determining nucleic acid sequence of amplification products in the set with homogeneous amplification products to determine a first number of assay samples which contain the first sequence and a second number of assay samples which contain the second sequence

Ruano was amplifying a 770 bp fragment in the intergenic region between ψβ and δ, which fragment contained a polymorphic site (figure 1). One allele constitutes a TaqI restriction site, while the other does not (*id.*). Thus, the sequence of the SMD amplification product could be either of these two sequences, and Ruano determined the correct sequence of the SMD amplification product in each of the sample containing

homogeneous products (i.e. lanes 5, 6, 7 and 10 of figure 3a) by analyzing TaqI digests of the amplification products (figure 3b). Ruano thereby determined that 2 of the 4 samples yielding homogeneous amplification products comprised the TaqI-positive allele, while the other 2 comprised the TaqI-negative allele.

Ruano did not expressly teach:

comparing the first number to the second number to ascertain a ratio which reflects the composition of the mixed population;

identifying a mutation in the mixed population if a statistically significant fraction of samples comprises the second sequence.

It would have been *prima facie* obvious to one of ordinary skill in the art at the time the invention was made to compare the number of assay samples yielding the first sequence to the number of samples yielding the second sequence to arrive at a ratio, and to identify the presence of a mutation (note that either the TaqI-positive or TaqI-negative allele can be considered "wild-type" while the other can be considered the "mutation") is a statistically significant fraction of samples comprised the second sequence. This would have been self-evident by merely looking at the results in Ruano's figure 3b. It was clearly evident that 50% (a statistically significant fraction) of the samples yielding an amplification product had the second sequence. Thus, it would have been obvious for one of ordinary skill in the art to look at the results and make a mental comparison of the number of samples yielding each allele and think: "Hmmm. Fifty percent of the samples are $TaqI^+$ and fifty percent are $TaqI^-$, so both alleles [and hence the "mutation"] are present." This simple assessment arrives at the limitations:

comparing the first number to the second number to ascertain a ratio which reflects the composition of the mixed population;

identifying a mutation in the mixed population if a statistically significant fraction of samples comprises the second sequence.

With regard to claim 74, Ruano's dilution was designed to give, on average, one molecule of template per sample; see page 6297, column 2, line 6+. Ruano did not teach a desire to achieve an average of 0.5 molecules of template per sample.

Ruano did not teach at least 15 samples.

Stephens taught theoretical considerations for single molecule dilution of nucleic acid for the purposes of trying to amplify single template molecules. In Table 1 (page 1150), Stephens estimates the probability of success (defined as having one or more of *v* vials having one but not both haplotypes present; see sentence spanning columns 1-2, page 1150). According to this table, for a given dilution (i.e. DNA concentration in haploid equivalents), the probability of success increases with an increasing number of samples (i.e. vials, *v*). Also according to this table, while a DNA concentration of 1 is optimal, a DNA concentration of 0.5 still has a very high probability of success. Thus, Stephens establishes that both the number of samples used and the dilution (i.e. the average number of template molecules per sample) are results-effective variables. Note that Stephens' table is based on theoretical calculations, so while his figures would have provided a reasonable place to start, some actual empirical optimization would have been warranted. For this reason, claim 74 is *prima facie* obvious on the basis that it is *prima facie* obvious to optimize conditions known in the prior art that were results-

effective variables (in this case, the number of samples and the number of template molecules per sample; see MPEP 2144.05(II)).

Response to Arguments

Applicant's arguments filed 09/25/2013 have been fully considered but they are not persuasive. The arguments made on page 9 with regard to previous claim 50 would be applicable to the instant rejection of claim 74. Applicant argues:

> Stephens is cited as teaching in Table 1 the probability of success at having one or more vials having one but not both haplotypes in it. Stephens teaches that for 10 vials and 1 haploid equivalent in a vial, as used by Ruano, one has a 0.9985 chance of success. In fact, of the choices shown in Table 1, it appears that Ruano used the optimal conditions. Contrary to the assertion of the Patent and Trademark Office, Table 1 would not have motivated one of ordinary skill in the art to use even more assay vials. A 0.9985 chance of success would be considered to be quite high and increasing the number of vials would likely yield a very small increase. Stephens, contrary to the Patent and Trademark Office's assertion, would lead one of skill in the art to do exactly what Ruano had done—10 samples and single molecule dilution. No modification of the method of Ruano would have been suggested.
>
> Similarly, contrary to the assertion of the Patent and Trademark Office, Stephens would not motivate one of skill in the art to modify Ruano's teaching of single molecule dilution to use 0.5 molecules, as recited in claim 50. Inspection of Stephens' Table 1 would lead one of skill in the art to realize that modifying Ruano's method in that way would lead to a diminished success rate.
>
> One of ordinary skill in the art would not have found Stephens' teaching to be motivational for any empirical optimization. Stephens explicitly taught the theoretical underpinnings for the parameters that Ruano chose—they were the optimum for the method that Ruano was performing. Once optimized, those of skill in the art are not likely to seek variations.

This argument is not persuasive. Stephens' Table 1 shows the theoretical estimates of probability of success without taking shearing into account. But shearing, as would inevitably occur in isolating genomic DNA, is a source of potential error (Stephens, page 1150, column 1, last paragraph). As shown in Figure 2 of Stephens, the number of vials needed to maximize the probability of success $P(S_x)$ varies depending on the concentration of DNA *and* on the amount of breakage (shearing) of the DNA:

Figure 2 Number of vials needed to maximize $P(S_x)$ for DNA concentration in the range of 0.2–3.0 haploid equivalents and for breakage in the range of 5%–15% of target chromosomes. Black bar (■) = 5% broken; beaded bar (▦) = 10% broken; halftone bar (▩) = 15% broken.

It is clearly seen that, whereas 10 vials might be optimal for a DNA concentration of 1 haploid equivalent per vial at breakage rate of 15%, slightly more vials would be need to maximize the probability of success for breakage at 5%, or at lower DNA concentrations. In short, the difference between Ruano and claim 74 is a DNA concentration of 1 haploid equivalent for Ruano, versus 0.5 for claim 74, and 10 vials for Ruano, versus 15 vials for claim 74. Stephens' figure 2 demonstrates these differences were obvious; Applicant's claim 74 does not warrant a patent because it requires a

different, previously taught DNA concentration and a different, previously taught number of vials.

Conclusion

Any inquiry concerning this communication or earlier communications from the examiner should be directed to SAMUEL WOOLWINE whose telephone number is (571)272-1144. The examiner can normally be reached on Mon-Fri 9:00am-5:00pm.

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.

/Samuel Woolwine/
Primary Examiner

Search Notes	Application/Control No.	Applicant(s)/Patent Under Reexamination
	13071105	VOGELSTEIN ET AL.
	Examiner	Art Unit
	SAMUEL WOOLWINE	1637

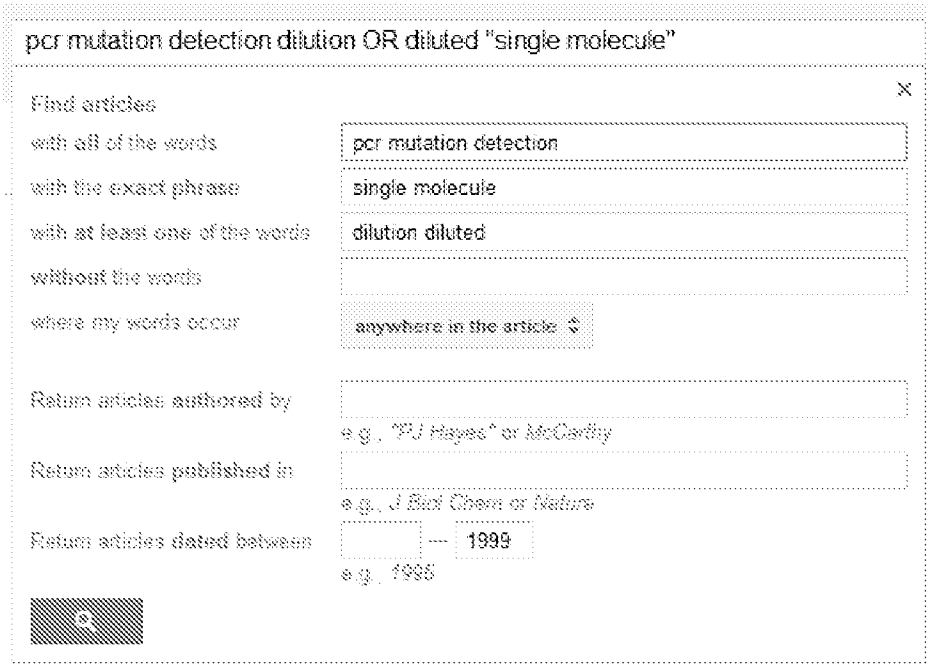
CPC- SEARCHED		
Symbol	Date	Examiner

CPC COMBINATION SETS - SEARCHED		
Symbol	Date	Examiner

US CLASSIFICATION SEARCHED			
Class	Subclass	Date	Examiner

SEARCH NOTES		
Search Notes	Date	Examiner
Inventor name/keyword search in EAST, Google Scholar.	10/04/2012	SCW
Update search in STN	06/15/2013	SCW
Update search in Google Scholar and EAST	12/28/2013	SCW

INTERFERENCE SEARCH			
US Class/ CPC Symbol	US Subclass / CPC Group	Date	Examiner
			SCW


pcr mutation detection dilution OR diluted "single molecule"
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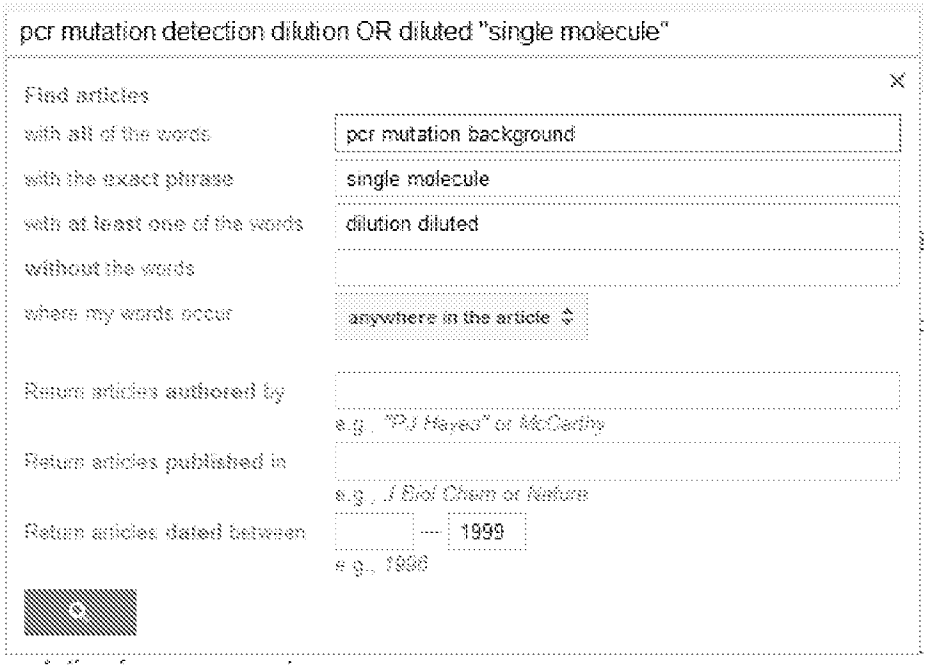
pcr mutation detection dilution OR diluted "single molecule"
Find articles
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with the exact phrase
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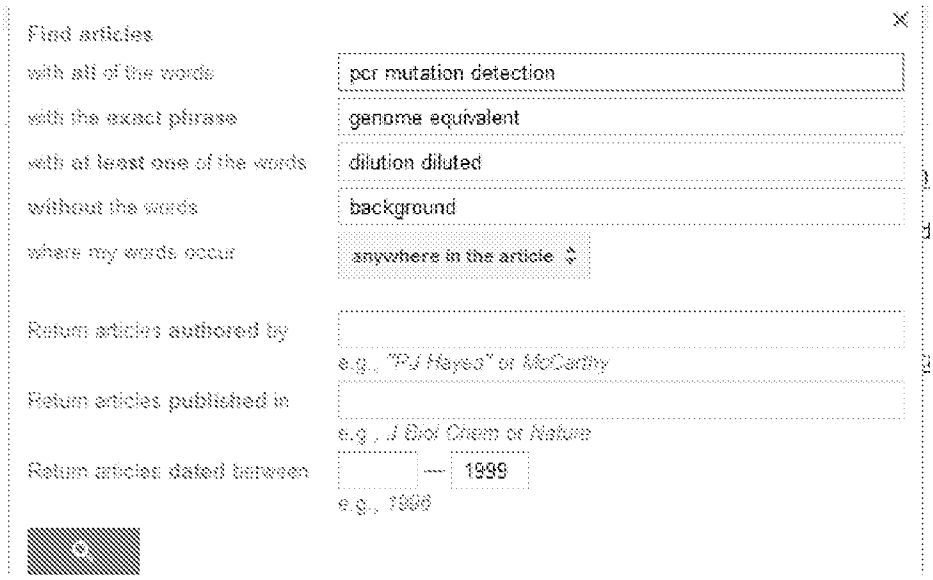
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CONFIRMATION NO. 3361

SERIAL NUMBER	FILING or 371(c) DATE RULE	CLASS	GROUP ART UNIT	ATTORNEY DOCKET NO.
13/071,105	03/24/2011	435	1637	001107.00866

APPLICANTS

INVENTORS

Bert VOGELSTEIN, Baltimore, MD;
Kenneth W. KINZLER, Baltimore, MD;

**** CONTINUING DATA ***************************

This application is a CON of 12/617,368 11/12/2009 PAT 7915015
which is a CON of 11/709,742 02/23/2007 PAT 7824889
which is a CON of 10/828,295 04/21/2004 ABN
which is a DIV of 09/981,356 10/12/2001 PAT 6753147
which is a CON of 09/613,826 07/11/2000 PAT 6440706
which claims benefit of 60/146,792 08/02/1999

**** FOREIGN APPLICATIONS ***************************

**** IF REQUIRED, FOREIGN FILING LICENSE GRANTED ****

04/19/2011

Foreign Priority claimed ☐ Yes ☑ No 35 USC 119(a-d) conditions met ☐ Yes ☐ No Verified and Acknowledged /SAMUEL C WOOLWINE/ Examiner's Signature	☐ Met after Allowance Initials	STATE OR COUNTRY	SHEETS DRAWINGS	TOTAL CLAIMS	INDEPENDENT CLAIMS
		MD	7	48	5

ADDRESS

Banner & Witcoff, Ltd.
Attorneys for client 001107
1100 13th Street N.W.
Suite 1200
Washington, DC 20005-4051
UNITED STATES

TITLE

Digital Amplification

FILING FEE RECEIVED		
4436	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

EAST Search History

EAST Search History (Prior Art)

Ref #	Hits	Search Query	DBs	Default Operator	Plurals	Time Stamp
L1	4	("6440706" "6753147" "7824889" "7915015").pn.	USPAT	OR	OFF	2013/12/28 18:15
L2	1	"071105".ap. and vogelstein.in.	US-PGPUB; USPAT	OR	OFF	2013/12/28 18:56
L3	193438	(distributing distribute distributed diluting dilute diluted dilution aliquotting aliquot aliquotted dividing divide divided apportioning apportion apportioned) near10 sample	US-PGPUB; USPAT	OR	ON	2013/12/28 18:59
L4	60236	l3 and pcr	US-PGPUB; USPAT	OR	ON	2013/12/28 18:59
L5	25978	l4 and (single near5 (molecule template))	US-PGPUB; USPAT	OR	ON	2013/12/28 18:59
L6	9046	l4 and (less near5 (genome molecule template))	US-PGPUB; USPAT	OR	ON	2013/12/28 19:00
L7	925	l4 and (equivalent near5 (genome haploid))	US-PGPUB; USPAT	OR	ON	2013/12/28 19:00
L8	29128	l5 l6 l7	US-PGPUB; USPAT	OR	ON	2013/12/28 19:01
L9	6154	l8 and (single adj1 (molecule nucleic template fragment target))	US-PGPUB; USPAT	OR	ON	2013/12/28 19:01
L10	2234	l8 and (single adj1 (molecule nucleic template fragment target)) same (pcr amplification amplified amplifying)	US-PGPUB; USPAT	OR	ON	2013/12/28 19:02
L11	864	l10 and ((detection detecting detect) near5 (mutant mutation mutated allele))	US-PGPUB; USPAT	OR	ON	2013/12/28 19:03
L12	42	l11 and (@ad<"19990802" @pd<"19990802")	US-PGPUB; USPAT	OR	ON	2013/12/28 19:04
L13	639	"digital pcr"	US-PGPUB; USPAT	OR	ON	2013/12/28 19:11
L14	2421	(single adj1 molecule) near5 (pcr amplification dilution analysis)	US-PGPUB; USPAT	OR	ON	2013/12/28 19:44
L15	1829	l14 and pcr	US-PGPUB; USPAT	OR	ON	2013/12/28 19:44
L16	104	l10 AND ((C12Q1/6806).CPC.)	US-	OR	ON	2013/12/28

			PGPUB; USPAT			20:08
L17	80	l16 and (single adj1 (molecule template))	US-PGPUB; USPAT	OR	ON	2013/12/28 20:09
L18	6	l17 and (@ad<"19990802" @pd<"19990802")	US-PGPUB; USPAT	OR	ON	2013/12/28 20:09
L19	6	pcr AND ((C12Q2527/146).CPC.)	US-PGPUB; USPAT	OR	ON	2013/12/28 20:13
L20	6	((C12Q2527/146).CPC.)	US-PGPUB; USPAT	OR	ON	2013/12/28 20:13

EAST Search History (Interference)

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12/28/2013 8:16:07 PM
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INFORMATION DISCLOSURE STATEMENT BY APPLICANT

(Not for submission under 37 CFR 1.99)

Application Number	13071105
Filing Date	2011-03-24
First Named Inventor	Bert VOGELSTEIN
Art Unit	1637
Examiner Name	Samuel C. Woolwine
Attorney Docket Number	001107.00866

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Examiner Initial*	Cite No	Patent Number	Kind Code[1]	Issue Date	Name of Patentee or Applicant of cited Document	Pages,Columns,Lines where Relevant Passages or Relevant Figures Appear
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/S.W./	1	0643140	EP	A1	1995-03-15	Yamamoto, et al.		☐

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	Examiner Name	Samuel C. Woolwine
	Attorney Docket Number	001107.00866

/S.W./	1	Examiner Requisition issued dated April 12, 2013 issued by the Canadian Intellectual Property Office in Canadian Application No. 2,756,675	☐
/S.W./	2	Piatek, A. et al., "Molecular beacon sequence analysis for detecting drug resistance in Mycobacterium tuberculosis", Nature Biotechnology, 16(4), Pages 359-363, April 1, 1998.	☐

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Examiner Signature	/Samuel Woolwine/	Date Considered	12/13/2013

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[1] See Kind Codes of USPTO Patent Documents at www.USPTO.GOV or MPEP 901.04. [2] Enter office that issued the document, by the two-letter code (WIPO Standard ST.3). [3] For Japanese patent documents, the indication of the year of the reign of the Emperor must precede the serial number of the patent document. [4] Kind of document by the appropriate symbols as indicated on the document under WIPO Standard ST.16 if possible. [5] Applicant is to place a check mark here if English language translation is attached.

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OR

☐ That no item of information contained in the information disclosure statement was cited in a communication from a foreign patent office in a counterpart foreign application, and, to the knowledge of the person signing the certification after making reasonable inquiry, no item of information contained in the information disclosure statement was known to any individual designated in 37 CFR 1.56(c) more than three months prior to the filing of the information disclosure statement. See 37 CFR 1.97(e)(2).

☐ See attached certification statement.

☒ The fee set forth in 37 CFR 1.17 (p) has been submitted herewith.

☐ A certification statement is not submitted herewith.

SIGNATURE

A signature of the applicant or representative is required in accordance with CFR 1.33, 10.18. Please see CFR 1.4(d) for the form of the signature.

Signature	/Sarah A. Kagan/	Date (YYYY-MM-DD)	2013-07-12
Name/Print	Sarah A. Kagan	Registration Number	32141

This collection of information is required by 37 CFR 1.97 and 1.98. 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 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: Commissioner for Patents, P.O. Box 1450, Alexandria, VA 22313-1450.**

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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.

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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.

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First Named Inventor	Bert Vogelstein
Art Unit	1637
Examiner Name	Samuel C. Woolwine
Attorney Docket Number	001107.00866

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Examiner Initial*	Cite No	Patent Number	Kind Code[1]	Issue Date	Name of Patentee or Applicant of cited Document	Pages,Columns,Lines where Relevant Passages or Relevant Figures Appear
	1	5928907		1999-07-27	WOUDENBERG ET AL.	

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	Attorney Docket Number	001107.00866

	1	Schwab, "Amplification of oncogenes in human cancer cells," Bioessays 20(6): 473-479 (1998)	☐
	2	Non-Final Office Action issued in related U.S. Reexamination Application No. 90/012,894, mailed November 27, 2013	☐
	3	Jeffreys et al., "Amplification of human minisatellites by the polymerase chain reaction: towards DNA fingerprinting of single cells," Nucl. Acids. Res., vol. 16, no. 23, pages 10953-10971 (1988)	☐
	4	Non-Final Office Action issued in related U.S. Reexamination Application No. 90/012,895, mailed November 27, 2013	☐
	5	Non-Final Office Action issued in related U.S. Reexamination Application No. 90/012,896, mailed November 27, 2013	☐
	6	Supplemental Joint Claim Construction Statement filed in Civil Action No. 12-cv-1173-CCE-JEP on October 28, 2013 (filed with exhibits A, B, and C)	☐
	7	Defendants' Responsive Claim Construction Brief filed in Case No. 1:12-CV-1173 on November 26, 2013	☐
	8	Deposition of David Sherman, Ph.D., dated October 17, 2013	☐
	9	Supplemental Joint Claim Construction Statement Exhibit C filed in Civil Action No. 12-cv-1173-CCE-JEP on October 28, 2013 (filed with Supplemental Joint Claim Construction Statement filed in Civil Action No. 12-cv-1173-CCE-JEP on October 28, 2013)	☐
	10	Plaintiffs' Responsive Claim Construction Brief filed in filed in Civil Action No. 12-cv-1173-CCE-JEP on November 26, 2013	☐
	11	Plaintiffs' Proposed Construction of Disputed Terms, Supporting Evidence, and Rebuttal Evidence Exhibit B, filed in filed in Civil Action No. 12-cv-1173-CCE-JEP on October 28, 2013 (filed with Supplemental Joint Claim Construction Statement filed in Civil Action No. 12-cv-1173-CCE-JEP on October 28, 2013)	☐

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	Attorney Docket Number	001107.00866

	12	Declaration of David H. Sherman in Support of Esoterix Genetic Laboratories' Claim Construction Brief filed in Civil Action Nos. 12-cv-411-CCE-JEP and 12-cv-1173-CCE-JEP, executed September 27, 2013	☐
	13	Defendants' Opening Claim Construction Brief filed in Case No. 1:12-CV-1173 on November 5, 2013	☐
	14	Exhibit A filed in Civil Action No. 12-cv-1173-CCE-JEP on October 28, 2013 (filed with Supplemental Joint Claim Construction Statement filed in Civil Action No. 12-cv-1173-CCE-JEP on October 28, 2013)	☐
	15	Plaintiffs' Opening Claim Construction Brief filed in Civil Action No. 12-cv-1173-CCE-JEP on November 5, 2013	☐

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☐ A certification statement is not submitted herewith.

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Signature	/Sarah A. Kagan/	Date (YYYY-MM-DD)	2014-02-05
Name/Print	Sarah A. Kagan	Registration Number	32141

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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.

Electronic Patent Application Fee Transmittal

Application Number:	13071105
Filing Date:	24-Mar-2011
Title of Invention:	Digital Amplification
First Named Inventor/Applicant Name:	Bert VOGELSTEIN
Filer:	Sarah Anne Kagan./Jennifer Hazzard
Attorney Docket Number:	001107.00866

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:	18127710
Application Number:	13071105
International Application Number:	
Confirmation Number:	3361
Title of Invention:	Digital Amplification
First Named Inventor/Applicant Name:	Bert VOGELSTEIN
Customer Number:	11332
Filer:	Sarah Anne Kagan./Jennifer Hazzard
Filer Authorized By:	Sarah Anne Kagan.
Attorney Docket Number:	001107.00866
Receipt Date:	06-FEB-2014
Filing Date:	24-MAR-2011
Time Stamp:	07:53:51
Application Type:	Utility under 35 USC 111(a)

Payment information:

Submitted with Payment	yes
Payment Type	Deposit Account
Payment was successfully received in RAM	$180
RAM confirmation Number	6137
Deposit Account	190733
Authorized User	

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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)	IDSSB08.PDF	613164 23d626e3d28e22d23afca52ad1a449d35144c9b8	no	5
Warnings:					
Information:					
2	Non Patent Literature	SCHWAB.PDF	1139589 be2723f39751c1a7c2d14373817f02dc37704927	no	7
Warnings:					
Information:					
3	Non Patent Literature	2013nov27-US-OA-1.PDF	1116726 365a789f794675eb1686adc20b9c4013c1824d74	no	22
Warnings:					
Information:					
4	Non Patent Literature	JEFFREYS.PDF	1002779 e53bdb2b1de1c0c7c560c2c8d129d00fcf6a1610	no	19
Warnings:					
Information:					
5	Non Patent Literature	2013nov27-US-OA-2.PDF	621612 88766c466c951d2ce5674b40cc110b13214b0113	no	16
Warnings:					
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6	Non Patent Literature	2013nov27-US-OA-3.PDF	552874 99022b60182b5980cb70ac45f9084133f20184ed	no	15
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7	Non Patent Literature	NPL-6.PDF	270942 0887199e2ff5a5b2369181b69f9eb8f7e791319f	no	7
Warnings:					
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8	Non Patent Literature	NPL-7.PDF	1602260 ee19b3ccbece421ea82e6ddf202bdd44a89ff9b1	no	31

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Information:					
9	Non Patent Literature	NPL-8.PDF	8816248 87febcde97e99964ba5c58c565a2ec5578200f04	no	110
Warnings:					
Information:					
10	Non Patent Literature	NPL-9.PDF	2100308 de213cf9cccd178b58f2fd9916b45181b55d4c8c	no	26
Warnings:					
Information:					
11	Non Patent Literature	NPL-10.PDF	1253136 7ea19c50e28d926e06363bac66ae4d3c008f88f4	no	31
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12	Non Patent Literature	NPL-11.PDF	1218520 365cfc46661dd227026b469b6c69abfb5f8ce260	no	16
Warnings:					
Information:					
13	Non Patent Literature	NPL-12.PDF	1780447 b0b9a86407c5f8f21bcfb0749c12b4e7289d8c55	no	38
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Information:					
14	Non Patent Literature	NPL-13.PDF	1679774 498736b96ba6da6608f127a55f63fd34fd6e08d4	no	31
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Information:					
15	Non Patent Literature	NPL-14.PDF	450206 f6e06a26d55df5f0f787773f7f72488eeea4330a	no	6
Warnings:					
Information:					
16	Non Patent Literature	NPL-15.PDF	1375032 212576e91f8996d58026ae34164796a5cf5d59c6	no	31
Warnings:					
Information:					
17	Fee Worksheet (SB06)	fee-info.pdf	30251 f9fb0106e5e1fbc14c25f73e519c48cb31eba89a	no	2

Warnings:	
Information:	
Total Files Size (in bytes):	25623868

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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.

PATENT

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re Application of	)	
	)	
Bert VOGELSTEIN et al.	)	Examiner: WOOLWINE, Samuel C.
	)	
Serial No. 13/071,105	)	
	)	Group Art Unit: 1637
	)	
Filed: March 24, 2011	)	Confirmation No. 3361
	)	
For: **DIGITAL AMPLIFICATION**	)	Atty. Dkt. No. 001107.00866

RESPONSE TO OFFICE ACTION

Commissioner of Patents
P.O. Box 1450
Alexandria, VA 22313-1450

Sir:

In response to the office action mailed December 30, 2013, applicants request entry of the amendment and reconsideration of the patentability of the claims in view of the remarks.

No petition for extension of time accompanies this submission. The Commissioner is authorized to charge any fees which may be required or credit any overpayment to our Deposit Account 19-0733.

IN THE CLAIMS

Please replace the following claim set for that currently of record.

1. -48. (Cancelled)

49. (Previously presented) A method for detecting quantity of a genetic sequence in a mixed population of human genomic nucleic acid sequences comprising at least a first and a second human genomic sequence, wherein the first sequence is a wild-type sequence of an allele and a second sequence is a mutant sequence of the allele, comprising:

distributing or diluting a mixed population of cell-free, human genomic nucleic acid template molecules from a sample in which the fraction of mutant alleles is less than 20 %, into a set comprising at least fifteen assay samples such that said at least fifteen assay samples each comprises less than ten template molecules;

amplifying the template molecules in the assay samples, wherein an assay sample with a single template molecule forms homogeneous amplification products in the assay sample;

analyzing by determining nucleic acid sequence of amplification products in the assay samples of the set with homogeneous amplification products to determine a first number of assay samples in the set which contain the first sequence and a second number of assay samples in the set which contain the second sequence;

comparing the first number to the second number to ascertain a ratio which reflects the composition of the mixed population;

identifying a mutation in the mixed population if a statistically significant fraction of assay samples comprises the second sequence.

50. (Previously Presented) The method of claim 49 wherein the assay samples of the set have on average 0.5 molecules of template.

51. (Previously Presented) The method of claim 49 wherein between 0.1 and 0.9 of the assay samples yield an amplification product.

52. (Previously Presented) The method of claim 49 wherein the mixed population of nucleic acid sequences is distributed or diluted to a single template molecule level in the assay samples.

53. (Previously presented) The method of claim 49 wherein the mixed population of nucleic acid sequences is from a body sample.

54. (Previously presented) The method of claim 53 wherein the mixed population of nucleic acids sequences is from a body sample selected from the group consisting of stool, blood, and lymph nodes.

55. (Cancelled)

56. (Previously Presented) The method of claim 49 wherein the mixed population of nucleic acids sequences is distributed or diluted such that at least twenty assay samples comprise less than ten template molecules.

57. (Previously Presented) The method of claim 49 wherein the mixed population of nucleic acids sequences is distributed or diluted such that at least twenty-five assay samples comprise less than ten template molecules.

58. (Previously Presented) The method of claim 49 wherein the mixed population of nucleic acids sequences is distributed or diluted such that at least thirty assay samples comprise less than ten template molecules.

59. (Previously Presented) The method of claim 49 wherein the mixed population of nucleic acids sequences is distributed or diluted such that at least forty assay samples comprise less than ten template molecules.

60. (Previously Presented) The method of claim 49 wherein the mixed population of nucleic acids sequences is distributed or diluted such that at least fifty assay samples comprise less than ten template molecules.

61. (Previously Presented) The method of claim 49 wherein the mixed population of nucleic acids sequences is distributed or diluted such that at least seventy-five assay samples comprise less than ten template molecules.

62. (Previously Presented) The method of claim 49 wherein the mixed population of nucleic acids sequences is distributed or diluted such that at least one hundred assay samples comprise less than ten template molecules.

63. (Previously Presented) The method of claim 49 wherein the mixed population of nucleic acids sequences is distributed or diluted such that at least five hundred assay samples comprise less than ten template molecules.

64. (Previously Presented) The method of claim 49 wherein the mixed population of nucleic acids sequences is distributed or diluted such that at least one thousand assay samples comprise less than ten template molecules.

65. (Previously presented) The method of claim 49 wherein the mixed population of nucleic acids sequences is distributed or diluted such that at least one thousand assay samples are distributed or diluted to a single template molecule level.

66. (Previously Presented) The method of claim 49 wherein the mixed population of nucleic acids sequences is distributed or diluted such that at least one thousand assay samples has on average 0.5 molecules of template.

67. (Previously Presented) The method of claim 49 wherein the mixed population of nucleic acids sequences is distributed or diluted such that between 0.1 and 0.9 of at least one thousand assay samples yield an amplification product.

68. (Previously Presented) The method of claim 49 wherein the mixed population of nucleic acids sequences is distributed or diluted such that one half of at least one thousand assay samples have one template molecule.

69. (Previously presented) The method of claim 49 wherein the mutation is a somatic mutation.

70. (Previously presented) The method of claim 49 wherein the mutation is a cancer gene mutation.

71. (Previously presented) The method of claim 49 wherein the template molecules are from a population of cells which are not purely tumor cells.

72. (Previously presented) The method of claim 49 wherein between 1% and 10 % of the alleles in said human genomic nucleic acid template molecules are the mutant sequence of the allele.

73. (Previously presented) The method of claim 49 wherein the mixed population of nucleic acid sequences is from a tissue.

74. (Currently amended) A method for detecting quantity of a genetic sequence in a mixed population of human genomic nucleic acid sequences comprising at least a first and a second human genomic sequence, wherein the first sequence is a wild-type sequence of an allele and a second sequence is a mutant sequence of the allele, comprising:

distributing or diluting a mixed population of cell-free, human genomic nucleic acid template molecules into a set comprising at least fifteen assay samples such that said at least fifteen assay samples ~~comprises~~ comprise an average of 0.5 molecules of template~~.~~;

amplifying the template molecules in the assay samples, wherein an assay sample with a single template molecule forms homogeneous amplification products in the assay sample;

analyzing by determining nucleic acid sequence of amplification products in the assay samples of the set with homogeneous amplification products to determine a first number of assay samples in the set which contain the first sequence and a second number of assay samples in the set which contain the second sequence;

comparing the first number to the second number to ascertain a ratio which reflects the composition of the mixed population;

identifying a mutation in the mixed population ~~if~~ when a statistically significant fraction of assay samples comprises the second sequence.

75. (New) The method of claim 74 wherein the step of analyzing determines sequence of nucleic acid consisting of the wild-type allele, the mutant allele, or both alleles.

76. (New) The method of claim 74 wherein the alleles are at a single chromosomal locus and nucleic acids consisting of all or part of the locus are analyzed in the step of analyzing.

77. (New) The method of claim 74 wherein nucleic acid sequence is determined at a single locus.

78. (New) The method of claim 74 wherein the mixed population of nucleic acids sequences is from a body sample selected from the group consisting of stool, blood, and lymph nodes.

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Remarks

The rejection of claim74 under § 103(a)

Claims 74 stands rejected as unpatentable over Ruano (*Proc. Natl. Acad. Sciences USA 87:*6296-6300 (1990) in view of Stephens (*Am. J. Hum. Gen. 46:*1149-1155(1990).

Step 1 of claim 74 recites "distributing or diluting a mixed population of cell-free, human genomic nucleic acid template molecules into a set comprising at least fifteen assay samples such that said at least fifteen assay samples comprises an average of 0.5 molecules of template." Ruano is cited as teaching similarly for a set of at least ten assay samples comprising less than ten template molecules each. The rejection points to figure 3a of Ruano which shows ten samples having 1 template copy per sample. The rejection acknowledges that Ruano did not teach an average of 0.5 molecules of template per sample nor did Ruano teach making a set of assay samples comprising at least 15 samples.

Further, Ruano is cited as teaching formation of a homogeneous amplification product because of the single template molecule in each sample. This is said to fulfill step 2 of claim 74, *i.e.,* "amplifying the template molecules in the assay samples, wherein an assay sample with a single template molecule forms homogeneous amplification products in the assay sample."

Ruano is further cited as determining which of two polymorphic sequences was present in each of the lanes, thus fulfilling step 3 of claim 74.

The rejection acknowledges that Ruano does not expressly teach steps 4 and 5, *i.e.,* comparing and identifying a mutation. But these steps would allegedly have been *prima facie* obvious to anyone of ordinary skill in the art, because some people could do this calculation in their heads.

In order to bridge the gap from Ruano's smaller number of assay samples and higher average number of molecules per assay sample than the claimed method, the Patent and Trademark Office cites Stephens. Stephens is cited as teaching that an average number of templates of 1 per assay sample is optimal, but 0.5 still has a high probability of success, albeit sub-optimal. Stephens is also cited as teaching that probability of success increases with increasing sample number. Thus the rejection concludes that it would have been obvious for one of ordinary skill in the art to vary these two parameters and arrive at those that are claimed, *i.e.,* at least 15 assays samples in a set and an average of 0.5 templates per assay sample.

Previously applicants argued that Stephens's teachings (Table 1) would not have motivated one of skill in the art to change the Ruano parameters because the Ruano parameters were shown to be the optimum parameters and that varying them would have led to a diminution in rate of success according to the Stephens calculations. The Patent and Trademark Office did not find this persuasive because Stephens also teaches that shearing of DNA is a relevant parameter (Table 2). Stephens is cited as teaching that shearing affects the probability of success. The more breakage due to shearing that is assumed to occur, the more vials would be needed, the Patent and Trademark Office posits, rendering the increased number of vials in claim 74 obvious over the lower number used in Ruano.

However, Stephens's success, $P(S_x)$, is different than success for the method of claim 74. Stephens defines success as "having one or more of *v* vials having one but not both ***haplotypes***." Page 1150, sentence spanning columns 1 and 2 (emphasis added). See also the legend to Table 1. The haplotype that Stephens considers comprises two heterozygous loci, *A* and *B*. The unsheared fraction of DNA is intact between loci *A* and *B*, whereas the sheared fraction contains DNA with *A* and *B* on separate molecules. Page 1150, column 2, lines 19-25. But success for the method of claim 74 does not require that two loci remain on the same molecule of DNA. The method of claim 74 analyzes not haplotypes consisting of two or more loci on a single chromosome, but rather a single locus that has two allelic forms, *i.e.,* "the first sequence is a wild-type sequence of an allele and a second sequence is a mutant sequence of the allele." Typically the two allelic forms are on separate chromosomes (molecules) at the outset. Therefore, shearing would not have the effect of separating them onto separate molecules. Since Stephens teaches that shearing is a relevant parameter for successful detection of ***haplotypes,*** but the present invention does not detect haplotypes, one of ordinary skill in the art would not have looked to Stephens' Figure 2 for guidance in practicing the method of claim 74. The parameter would have no relevance to the method of claim 74 for ascertaining a ratio of a first allelic sequence to a second allelic sequence.

Since Figure 2 is of no relevance to the method of claim 74, one of skill in the art would refer to Table 1 of Stephens which shows that the conditions that were used by Ruano were the optimal conditions. One of ordinary skill in the art would therefore not have been motivated to modify the conditions of Ruano to achieve a diminished probability of success.

Even if one of ordinary skill in the art were motivated to combine the teachings of Stephens and Ruano, they would not have arrived at the method of claim 74 and new claims 75-78. Neither Stephens nor Ruano teaches steps 3-5 (analyzing, comparing, identifying) of the method. While the Patent and Trademark Office asserts that steps 3 and 4 (analyzing, comparing) would just happen *sua sponte* in the head of an ordinary artisan, no evidentiary support is provided for that assertion. Neither Stephens nor Ruano teaches these two steps. Even if they did teach steps 3 and 4, *arguendo,* certainly they do not teach step 6. Step 6 recites that a mutation is identified when a statistically significant faction of assay samples comprises the second sequence. In the case of Stephens and Ruano, however, who were performing haplotyping on polymorphic markers, no mutations were identified, only haplotypes consisting of known polymorphic markers.

Moreover, with regard to each of claims 75-77, these claims exclude multi-locus analysis as taught in Stephens and Ruano.

The method as recited in claim 74 is not obvious over the cited prior art. Please withdraw the rejection of claim 74 and process it, along with the allowed claims, for allowance.

Respectfully submitted,

Date: *27 March 2014*

By: */Sarah A. Kagan/*
Sarah A. Kagan
Registration No. 32,141

Banner & Witcoff, Ltd.
Customer No. 11332

Electronic Acknowledgement Receipt

EFS ID:	18605474
Application Number:	13071105
International Application Number:	
Confirmation Number:	3361
Title of Invention:	Digital Amplification
First Named Inventor/Applicant Name:	Bert VOGELSTEIN
Customer Number:	11332
Filer:	Sarah Anne Kagan.
Filer Authorized By:	
Attorney Docket Number:	001107.00866
Receipt Date:	27-MAR-2014
Filing Date:	24-MAR-2011
Time Stamp:	17:29:48
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	Amendment/Req. Reconsideration-After Non-Final Reject	00866.pdf	132193 f2a1373b0722f8a2ec415981b9d9712dd43db8f2	no	9

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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
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New International Application Filed with the USPTO as a Receiving Office
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11332 7590 07/07/2014

Banner & Witcoff, Ltd.
Attorneys for client 001107
1100 13th Street N.W.
Suite 1200
Washington, DC 20005-4051

EXAMINER
WOOLWINE, SAMUEL C

ART UNIT	PAPER NUMBER
1637	

DATE MAILED: 07/07/2014

APPLICATION NO.	FILING DATE	FIRST NAMED INVENTOR	ATTORNEY DOCKET NO.	CONFIRMATION NO.
13/071,105	03/24/2011	Bert VOGELSTEIN	001107.00866	3361

TITLE OF INVENTION: Digital Amplification

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	10/07/2014

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.

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Commissioner for Patents
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Alexandria, Virginia 22313-1450
or Fax (571)-273-2885

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11332 7590 07/07/2014
Banner & Witcoff, Ltd.
Attorneys for client 001107
1100 13th Street N.W.
Suite 1200
Washington, DC 20005-4051

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.

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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.
13/071,105	03/24/2011	Bert VOGELSTEIN	001107.00866	3361

TITLE OF INVENTION: Digital Amplification

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	10/07/2014

EXAMINER	ART UNIT	CLASS-SUBCLASS
WOOLWINE, SAMUEL C	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

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APPLICATION NO.	FILING DATE	FIRST NAMED INVENTOR	ATTORNEY DOCKET NO.	CONFIRMATION NO.
13/071,105	03/24/2011	Bert VOGELSTEIN	001107.00866	3361

11332 7590 07/07/2014

Banner & Witcoff, Ltd.
Attorneys for client 001107
1100 13th Street N.W.
Suite 1200
Washington, DC 20005-4051

EXAMINER	
WOOLWINE, SAMUEL C	

ART UNIT	PAPER NUMBER
1637	

DATE MAILED: 07/07/2014

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.

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. 13/071,105	Applicant(s) VOGELSTEIN ET AL.	
	Examiner SAMUEL WOOLWINE	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 *03/27/2014*.
   ☐ 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 *49-54,56-76 and 78*. 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 02/06/2014
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 _____.

/Samuel Woolwine/
Primary Examiner

EXAMINER'S AMENDMENT

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 a telephone interview with Sarah Kagan on 06/30/2014.

The application has been amended as follows:

Amend claims 49 and 74, lines 3-4 as follows:

"…wherein the first sequence is a sequence of a wild-type ~~sequence of an~~ allele of a locus and a second sequence is a sequence of a mutant ~~sequence of the~~ allele of the locus, comprising:…"

Amend claims 56-64 as follows:

"…assay samples each comprise less than ….."

Amend claim 74, step 1 as follows:

"…assay samples comprise an average of 0.5 molecules of template per assay sample…."

Cancel claim 77.

Response to Amendment

Claim Interpretation

For the purposes of this Office action, the Examiner construes the term "template" as recited in claims 49 and 74 to mean the particular locus of genomic

nucleic acid being amplified, regardless of whether the locus contains a mutant allele or a wild-type allele.

Response to Arguments

Applicant's arguments filed 03/27/2014 have been fully considered and found persuasive. Firstly, while Stephens taught both number of vials and number of molecules per vial to be results-effective variables, he also taught that the optimum situation for the purpose of haplotype analysis (i.e. Ruano's purpose) occurs with 10 vials and 1 template molecule (i.e. haploid equivalent) per vial; see Table 1. This is in fact what Ruano used.

Also to be understood from Stephens' teachings is that at 1 haploid equivalent per vial, 10 vials is sufficient for a probability of success that is indistinguishable from its theoretical maximum value (page 1152, last paragraph and see figure 3). Stephens provides no reasoning to drop the concentration to 0.5 equivalents per vial. In all cases, this provides a lesser probability of success as compared to 1 equivalent per vial; see Table 1.

Any inquiry concerning this communication or earlier communications from the examiner should be directed to SAMUEL WOOLWINE whose telephone number is (571)272-1144. The examiner can normally be reached on Mon-Fri 9:00am-5:00pm.

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.

/Samuel Woolwine/
Primary Examiner

Notice of References Cited	Application/Control No. 13/071,105	Applicant(s)/Patent Under Reexamination VOGELSTEIN ET AL.	
	Examiner SAMUEL WOOLWINE	Art Unit 1637	Page 1 of 3

U.S. PATENT DOCUMENTS

*		Document Number Country Code-Number-Kind Code	Date MM-YYYY	Name	Classification
	A	US-			
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FOREIGN PATENT DOCUMENTS

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	N					
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	P					
	Q					
	R					
	S					
	T					

NON-PATENT DOCUMENTS

*		Include as applicable: Author, Title Date, Publisher, Edition or Volume, Pertinent Pages)
	U	Final office action in related U.S. Reexamination application no. 90/012/894, mailed May 9, 2014
	V	Final office action in related U.S. Reexamination application no. 90/012/895, mailed May 9, 2014
	W	Final office action in related U.S. Reexamination application no. 90/012/896, mailed May 9, 2014
	X	Bischoff et al., "Single cell analysis demonstrating somatic mosaicism involving 11p in a patient with paternal isodisomy and Beckwith-Wiedemann syndrome", Human Molecular Genetics, Vol. 4, No. 3, 1995, 395-399

*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.

U.S. PATENT DOCUMENTS

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	R					
	S					
	T					

NON-PATENT DOCUMENTS

*		Include as applicable: Author, Title Date, Publisher, Edition or Volume, Pertinent Pages)
	U	Kalinina et al., "Nanoliter scale PCR with TaqMan detection," Nucl. Acids. Res.vol25, 1999-2004 (1997)
	V	Chou et al., "Prevention of pre-PCR mis-priming and primer dimerization improves low-copy-number amplifications," Nucleic Acids Res., 20(7): 1717-1723 (April11, 1992)
	W	Burg, et al., "Direct and sensitive detection of a pathogenic protozoan, Toxoplasma gondii, by polymerase chain reaction." J. Clin. Microbial. 27, 1787-1792 (1989)
	X	Trumper et at., "Single-Cell Analysis of Hodgkin and Reed-Sternberg Cells: Molecular Heterogeneity of Gene Expression and p53 Mutations," Blood, 81 : 3097-3115 (1993)

*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.

Notice of References Cited	Application/Control No. 13/071,105	Applicant(s)/Patent Under Reexamination VOGELSTEIN ET AL.	
	Examiner SAMUEL WOOLWINE	Art Unit 1637	Page 3 of 3

U.S. PATENT DOCUMENTS

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FOREIGN PATENT DOCUMENTS

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	N					
	O					
	P					
	Q					
	R					
	S					
	T					

NON-PATENT DOCUMENTS

*		Include as applicable: Author, Title Date, Publisher, Edition or Volume, Pertinent Pages)
	U	Kanzler et al., "Molecular Single Cell Analysis Demonstrates the Derivation of Peripheral Blood-Derived Cell Line (L 1236) From the Hodgkin/Reed Sternberg Cells of a Hodgkin's Lymphoma Patient," Blood, 87:3429-3436 (1996)
	V	Gravel et al., "Single-cell analysis of the t(14; 18)(q32;q21) chromosomal translocation in Hodgkin's disease demonstrates the absence of this translocation in neoplastic Hodgkin and Reed-Sternberg cells," Blood 91(8):2866-74 (Apr 15, 1998)
	W	Ponten et al., "Genomic analysis of single cells from human basal cell cancer using laser-assisted capture microscopy," Mutation Research Genomics 382, 45-55 (1997)
	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
L1	1316	((single solitary individual isolated discrete) adj2 (molecule template genome "haploid equivalent" "haploid equivalents" copy copies chromosome allele)) with (dilute dilution diluting diluted ((concentration) near3 (adjust adjusted adjusting)))	US-PGPUB; USPAT; USOCR; FPRS; EPO; JPO; DERWENT	OR	ON	2014/06/25 12:22
L2	1004	l1 and pcr	US-PGPUB; USPAT; USOCR; FPRS; EPO; JPO; DERWENT	OR	ON	2014/06/25 12:23
L3	35	l2 and (@ad<"19990802" @pd<"19990802")	US-PGPUB; USPAT; USOCR; FPRS; EPO; JPO; DERWENT	OR	ON	2014/06/25 12:23
L4	1844	((single solitary individual isolated discrete) adj2 (molecule template genome "haploid equivalent" "haploid equivalents" copy copies chromosome allele)) and ((proportion fraction ratio percentage) with (mutant mutated) with (wildtype "wild-type" "wild type"))	US-PGPUB; USPAT; USOCR; FPRS; EPO; JPO; DERWENT	OR	ON	2014/06/25 12:34
L5	1729	l4 and pcr	US-PGPUB; USPAT; USOCR; FPRS; EPO; JPO; DERWENT	OR	ON	2014/06/25 12:35
L6	1399	l5 and (dilution diluted dilute diluting ((adjust adjusted adjusts adjusting) near2 concentration))	US-PGPUB; USPAT; USOCR; FPRS; EPO; JPO; DERWENT	OR	ON	2014/06/25 12:36
L7	118	l6 and (@ad<"19990802" @pd<"19990802")	US-PGPUB; USPAT; USOCR; FPRS; EPO; JPO; DERWENT	OR	ON	2014/06/25 12:36
L8	23	l7 and (dilution diluted dilute diluting ((adjust adjusted adjusts adjusting) near2 concentration)) with (single solitary genome "haploid equivalent" allele chromosome individual discrete isolated)	US-PGPUB; USPAT; USOCR; FPRS; EPO; JPO; DERWENT	OR	ON	2014/06/25 12:39

EAST Search History (Interference)

<This search history is empty>

6/25/2014 12:48:35 PM

Doc code: IDS
Doc description: Information Disclosure Statement (IDS) Filed

PTO/SB/08a (01-10)
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.

INFORMATION DISCLOSURE STATEMENT BY APPLICANT

(Not for submission under 37 CFR 1.99)

Application Number	13071105
Filing Date	2011-03-24
First Named Inventor	Bert Vogelstein
Art Unit	1637
Examiner Name	Samuel C. Woolwine
Attorney Docket Number	001107.00866

U.S.PATENTS Remove

Examiner Initial*	Cite No	Patent Number	Kind Code[1]	Issue Date	Name of Patentee or Applicant of cited Document	Pages,Columns,Lines where Relevant Passages or Relevant Figures Appear
	1	5928907		1999-07-27	WOUDENBERG ET AL.	

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ALL REFERENCES CONSIDERED EXCEPT WHERE LINED THROUGH. /S.W./

INFORMATION DISCLOSURE STATEMENT BY APPLICANT (Not for submission under 37 CFR 1.99)		
	Application Number	13071105
	Filing Date	2011-03-24
	First Named Inventor	Bert Vogelstein
	Art Unit	1637
	Examiner Name	Samuel C. Woolwine
	Attorney Docket Number	001107.00866

	1	Schwab, "Amplification of oncogenes in human cancer cells," Bioessays 20(6): 473-479 (1998)	☐
	2	Non-Final Office Action issued in related U.S. Reexamination Application No. 90/012,894, mailed November 27, 2013	☐
	3	Jeffreys et al., "Amplification of human minisatellites by the polymerase chain reaction: towards DNA fingerprinting of single cells," Nucl. Acids. Res., vol. 16, no. 23, pages 10953-10971 (1988)	☐
	4	Non-Final Office Action issued in related U.S. Reexamination Application No. 90/012,895, mailed November 27, 2013	☐
	5	Non-Final Office Action issued in related U.S. Reexamination Application No. 90/012,896, mailed November 27, 2013	☐
	6	Supplemental Joint Claim Construction Statement filed in Civil Action No. 12-cv-1173-CCE-JEP on October 28, 2013 (filed with exhibits A, B, and C)	☐
	7	Defendants' Responsive Claim Construction Brief filed in Case No. 1:12-CV-1173 on November 26, 2013	☐
	8	Deposition of David Sherman, Ph.D., dated October 17, 2013	☐
	9	Supplemental Joint Claim Construction Statement Exhibit C filed in Civil Action No. 12-cv-1173-CCE-JEP on October 28, 2013 (filed with Supplemental Joint Claim Construction Statement filed in Civil Action No. 12-cv-1173-CCE-JEP on October 28, 2013)	☐
	10	Plaintiffs' Responsive Claim Construction Brief filed in filed in Civil Action No. 12-cv-1173-CCE-JEP on November 26, 2013	☐
	11	Plaintiffs' Proposed Construction of Disputed Terms, Supporting Evidence, and Rebuttal Evidence Exhibit B, filed in filed in Civil Action No. 12-cv-1173-CCE-JEP on October 28, 2013 (filed with Supplemental Joint Claim Construction Statement filed in Civil Action No. 12-cv-1173-CCE-JEP on October 28, 2013)	☐

ALL REFERENCES CONSIDERED EXCEPT WHERE LINED THROUGH. /S.W./

INFORMATION DISCLOSURE STATEMENT BY APPLICANT (Not for submission under 37 CFR 1.99)		
	Application Number	13071105
	Filing Date	2011-03-24
	First Named Inventor	Bert Vogelstein
	Art Unit	1637
	Examiner Name	Samuel C. Woolwine
	Attorney Docket Number	001107.00866

	12	Declaration of David H. Sherman in Support of Esoterix Genetic Laboratories' Claim Construction Brief filed in Civil Action Nos. 12-cv-411-CCE-JEP and 12-cv-1173-CCE-JEP, executed September 27, 2013	☐
	13	Defendants' Opening Claim Construction Brief filed in Case No. 1:12-CV-1173 on November 5, 2013	☐
	14	Exhibit A filed in Civil Action No. 12-cv-1173-CCE-JEP on October 28, 2013 (filed with Supplemental Joint Claim Construction Statement filed in Civil Action No. 12-cv-1173-CCE-JEP on October 28, 2013)	☐
	15	Plaintiffs' Opening Claim Construction Brief filed in Civil Action No. 12-cv-1173-CCE-JEP on November 5, 2013	☐

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EXAMINER SIGNATURE

Examiner Signature	/Samuel Woolwine/	Date Considered	06/22/2014

*EXAMINER: Initial if reference considered, whether or not citation is in conformance with MPEP 609. Draw line through a citation if not in conformance and not considered. Include copy of this form with next communication to applicant.

[1] See Kind Codes of USPTO Patent Documents at www.USPTO.GOV or MPEP 901.04. [2] Enter office that issued the document, by the two-letter code (WIPO Standard ST.3). [3] For Japanese patent documents, the indication of the year of the reign of the Emperor must precede the serial number of the patent document. [4] Kind of document by the appropriate symbols as indicated on the document under WIPO Standard ST.16 if possible. [5] Applicant is to place a check mark here if English language translation is attached.

ALL REFERENCES CONSIDERED EXCEPT WHERE LINED THROUGH. /S.W./

```
     (FILE 'HOME' ENTERED AT 12:51:49 ON 25 JUN 2014)

     FILE 'CAPLUS' ENTERED AT 12:52:30 ON 25 JUN 2014
L1         15963 SEA ABB=ON  PLU=ON  (DILUTION OR DILUTED OR DILUTING OR DILUTE
                 OR ((ADJUST OR ADJUSTED OR ADJUSTING)(2A)CONCENTRATION))(S)(SIN
                 GLE OR SOLITARY OR GENOME OR GENOMIC OR HAPLOID OR ALLELE OR
                 CHROMOSOME OR INDIVIDUAL OR DISCRETE OR ISOLATED)
L2             3 SEA ABB=ON  PLU=ON  L1 AND ((FRACTION OR RATIO OR PROPORTION
                 OR PERCENTAGE)(5A)(MUTANT OR MUTATED OR ALLELE)(5A)(WILDTYPE
                 OR "WILD-TYPE" OR "WILD TYPE"))
                 D 1-3 IBIB
L3           796 SEA ABB=ON  PLU=ON  L1 AND PCR
L4            97 SEA ABB=ON  PLU=ON  L3 AND (MUTANT OR MUTATED OR ALLELE)
L5            30 SEA ABB=ON  PLU=ON  L4 AND PD<19990802
                 D 1-30 IBIB
```

EAST Search History

EAST Search History (Prior Art)

Ref #	Hits	Search Query	DBs	Default Operator	Plurals	Time Stamp
L1	1797	(single adj1 (molecule template copy allele chromosome)) same (diluted dilution dilute diluting)	US-PGPUB; USPAT; USOCR; FPRS; EPO; JPO; DERWENT	OR	ON	2014/06/24 21:27
L2	881	(single adj1 (molecule template copy allele chromosome)) with (diluted dilution dilute diluting)	US-PGPUB; USPAT; USOCR; FPRS; EPO; JPO; DERWENT	OR	ON	2014/06/24 21:27
L3	536	((single adj1 (molecule template copy allele chromosome)) with (diluted dilution dilute diluting)) same (amplification amplifying amplified amplify pcr "polymerase chain")	US-PGPUB; USPAT; USOCR; FPRS; EPO; JPO; DERWENT	OR	ON	2014/06/24 21:28
L4	14	l3 and (@ad<"19990802" @pd<"19990802")	US-PGPUB; USPAT; USOCR; FPRS; EPO; JPO; DERWENT	OR	ON	2014/06/24 21:28
L5	7	((single adj1 (genome "haploid equivalent")) with (diluted dilution dilute diluting)) same (amplification amplifying amplified amplify pcr "polymerase chain")	US-PGPUB; USPAT; USOCR; FPRS; EPO; JPO; DERWENT	OR	ON	2014/06/24 21:44
L6	0	l5 and (@ad<"19990802" @pd<"19990802")	US-PGPUB; USPAT; USOCR; FPRS; EPO; JPO; DERWENT	OR	ON	2014/06/24 21:45

EAST Search History (Interference)

<This search history is empty>

6/24/2014 9:46:20 PM

EAST Search History

EAST Search History (Prior Art)

Ref #	Hits	Search Query	DBs	Default Operator	Plurals	Time Stamp
L1	1797	(single adj1 (molecule template copy allele chromosome)) same (diluted dilution dilute diluting)	US-PGPUB; USPAT; USOCR; FPRS; EPO; JPO; DERWENT	OR	ON	2014/06/24 21:27
L2	881	(single adj1 (molecule template copy allele chromosome)) with (diluted dilution dilute diluting)	US-PGPUB; USPAT; USOCR; FPRS; EPO; JPO; DERWENT	OR	ON	2014/06/24 21:27
L3	536	((single adj1 (molecule template copy allele chromosome)) with (diluted dilution dilute diluting)) same (amplification amplifying amplified amplify pcr "polymerase chain")	US-PGPUB; USPAT; USOCR; FPRS; EPO; JPO; DERWENT	OR	ON	2014/06/24 21:28
L4	14	l3 and (@ad<"19990802" @pd<"19990802")	US-PGPUB; USPAT; USOCR; FPRS; EPO; JPO; DERWENT	OR	ON	2014/06/24 21:28
L5	7	((single adj1 (genome "haploid equivalent")) with (diluted dilution dilute diluting)) same (amplification amplifying amplified amplify pcr "polymerase chain")	US-PGPUB; USPAT; USOCR; FPRS; EPO; JPO; DERWENT	OR	ON	2014/06/24 21:44
L6	0	l5 and (@ad<"19990802" @pd<"19990802")	US-PGPUB; USPAT; USOCR; FPRS; EPO; JPO; DERWENT	OR	ON	2014/06/24 21:45

EAST Search History (Interference)

Ref #	Hits	Search Query	DBs	Default Operator	Plurals	Time Stamp
L7	152	((single adj1 (molecule template copy allele chromosome)) with (diluted dilution dilute diluting)) same (amplification amplifying amplified amplify pcr "polymerase chain")	USPAT; UPAD	OR	ON	2014/06/24 21:47
L8	3	((single adj1 (molecule template copy allele chromosome)) with (diluted dilution dilute diluting)) same (amplification amplifying amplified amplify pcr "polymerase chain").clm.	USPAT; UPAD	OR	ON	2014/06/24 21:47
L9	1	((single adj1 (genome "haploid equivalent")) with (diluted dilution dilute diluting)) same (amplification amplifying amplified amplify pcr "polymerase chain")	USPAT; UPAD	OR	ON	2014/06/24 21:48

6/24/2014 9:48:38 PM

Issue Classification	Application/Control No.	Applicant(s)/Patent Under Reexamination
	13071105	VOGELSTEIN ET AL.
	Examiner	**Art Unit**
	SAMUEL WOOLWINE	1637

CPC							
Symbol						**Type**	**Version**
C12Q		1	/	6818		I	2013-01-01
C12Q		1	/	6851		F	2013-01-01
C12Q		1	/	686		I	2013-01-01
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			/				
			/				

CPC Combination Sets								
Symbol					**Type**	**Set**	**Ranking**	**Version**
			/					
			/					

NONE		**Total Claims Allowed:**	
(Assistant Examiner)	(Date)	28	
/SAMUEL WOOLWINE/ Primary Examiner.Art Unit 1637	06/30/2014	O.G. Print Claim(s)	O.G. Print Figure
(Primary Examiner)	(Date)	1	1A

Issue Classification	Application/Control No.	Applicant(s)/Patent Under Reexamination
	13071105	VOGELSTEIN ET AL.
	Examiner	**Art Unit**
	SAMUEL WOOLWINE	1637

US ORIGINAL CLASSIFICATION		INTERNATIONAL CLASSIFICATION									
CLASS	SUBCLASS	CLAIMED					NON-CLAIMED				
435	6.12	C	1	2	Q	1 / 68 (2006.0)	C	0	7	H	21 / 04

CROSS REFERENCE(S)					
CLASS	SUBCLASS (ONE SUBCLASS PER BLOCK)				
435	6.11				
536	24.3	24.31			

NONE		**Total Claims Allowed:**	
(Assistant Examiner)	(Date)	28	
/SAMUEL WOOLWINE/ Primary Examiner.Art Unit 1637	06/30/2014	O.G. Print Claim(s)	O.G. Print Figure
(Primary Examiner)	(Date)	1	1A

Issue Classification	Application/Control No.	Applicant(s)/Patent Under Reexamination
	13071105	VOGELSTEIN ET AL.
	Examiner	**Art Unit**
	SAMUEL WOOLWINE	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

NONE		**Total Claims Allowed:**	
(Assistant Examiner)	(Date)	28	
/SAMUEL WOOLWINE/ Primary Examiner.Art Unit 1637	06/30/2014	O.G. Print Claim(s)	O.G. Print Figure
(Primary Examiner)	(Date)	1	1A

Search Notes	Application/Control No.	Applicant(s)/Patent Under Reexamination
	13071105	VOGELSTEIN ET AL.
	Examiner	Art Unit
	SAMUEL WOOLWINE	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
Inventor name/keyword search in EAST, Google Scholar.	10/04/2012	SCW
Update search in STN	06/15/2013	SCW
Update search in Google Scholar and EAST	12/28/2013	SCW
Update search in EAST, STN	06/30/2014	SCW

INTERFERENCE SEARCH			
US Class/ CPC Symbol	US Subclass / CPC Group	Date	Examiner
	Text search in EAST	06/30/2014	SCW

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)

11332 7590 07/07/2014
Banner & Witcoff, Ltd.
Attorneys for client 001107
1100 13th Street N.W.
Suite 1200
Washington, DC 20005-4051

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.
13/071,105	03/24/2011	Bert VOGELSTEIN	001107.00866	3361

TITLE OF INVENTION: Digital Amplification

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	10/07/2014

EXAMINER	ART UNIT	CLASS-SUBCLASS
WOOLWINE, SAMUEL C	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 Banner & Witcoff, LTD.
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: The Johns Hopkins University

(B) RESIDENCE: (CITY and STATE OR COUNTRY): Baltimore, MD

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 190733 (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 /Sarah A. Kagan/ Date September 9, 2014

Typed or printed name Sarah A. Kagan Registration No. 32,141

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

Electronic Patent Application Fee Transmittal

Application Number:	13071105
Filing Date:	24-Mar-2011
Title of Invention:	Digital Amplification
First Named Inventor/Applicant Name:	Bert VOGELSTEIN
Filer:	Sarah Anne Kagan./Jennifer Hazzard
Attorney Docket Number:	001107.00866

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:				
Utility Appl Issue Fee	1501	1	960	960
Extension-of-Time:				

Description	Fee Code	Quantity	Amount	Sub-Total in USD($)
Miscellaneous:				
			Total in USD ($)	**960**

Electronic Acknowledgement Receipt

EFS ID:	20087489
Application Number:	13071105
International Application Number:	
Confirmation Number:	3361
Title of Invention:	Digital Amplification
First Named Inventor/Applicant Name:	Bert VOGELSTEIN
Customer Number:	11332
Filer:	Sarah Anne Kagan./Jennifer Hazzard
Filer Authorized By:	Sarah Anne Kagan.
Attorney Docket Number:	001107.00866
Receipt Date:	09-SEP-2014
Filing Date:	24-MAR-2011
Time Stamp:	15:06:03
Application Type:	Utility under 35 USC 111(a)

Payment information:

Submitted with Payment	yes
Payment Type	Deposit Account
Payment was successfully received in RAM	$960
RAM confirmation Number	13033
Deposit Account	190733
Authorized User	

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

Charge any Additional Fees required under 37 C.F.R. Section 1.16 (National application filing, search, and examination fees)

Charge any Additional Fees required under 37 C.F.R. Section 1.17 (Patent application and reexamination processing fees)

Charge any Additional Fees required under 37 C.F.R. Section 1.19 (Document supply fees)

Charge any Additional Fees required under 37 C.F.R. Section 1.20 (Post Issuance fees)

Charge any Additional Fees required under 37 C.F.R. Section 1.21 (Miscellaneous fees and charges)

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)	IssueFeeTransmittal_16910683.PDF	282094 60fa26d724207d7b5f42a2c92ebeda45f310e805	no	1
Warnings:					
Information:					
2	Fee Worksheet (SB06)	fee-info.pdf	30404 68fb782d72c6063f260d11384545b044b03aa6d2	no	2
Warnings:					
Information:					
		Total Files Size (in bytes):	312498		

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
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Alexandria, Virginia 22313-1450
www.uspto.gov

APPLICATION NO.	ISSUE DATE	PATENT NO.	ATTORNEY DOCKET NO.	CONFIRMATION NO.
13/071,105	10/14/2014	8859206	001107.00866	3361

11332 7590 09/24/2014

Banner & Witcoff, Ltd.
Attorneys for client 001107
1100 13th Street N.W.
Suite 1200
Washington, DC 20005-4051

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):

Bert VOGELSTEIN, Baltimore, MD;
Kenneth W. KINZLER, Baltimore, MD;