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(Also referred to as FORM PTO-1465)

**REQUEST FOR *EX PARTE* REEXAMINATION TRANSMITTAL FORM**

Address to:

**Mail Stop *Ex Parte* Reexam  
Commissioner for Patents  
P.O. Box 1450  
Alexandria, VA 22313-1450**Attorney Docket No.: **LT00831 REX**Date: **June 17, 2013**

1.  This is a request for *ex parte* reexamination pursuant to 37 CFR 1.510 of patent number 6,440,706 issued August 27, 2002. The request is made by:  
 patent owner.  third party requester.
2.  The name and address of the person requesting reexamination is:  
Life Technologies Corporation  
5791 Van Allen Way  
Carlsbad, CA 92008
3. Requester claims  small entity (37 CFR 1.27) or  micro entity status (37 CFR 1.29).
4.  a. A check in the amount of \$\_\_\_\_\_ is enclosed to cover the reexamination fee, 37 CFR 1.20(c)(1);  
 b. The Director is hereby authorized to charge the fee as set forth in 37 CFR 1.20(c)(1) to Deposit Account No. 503994;  
 c. Payment by credit card. Form PTO-2038 is attached; or  
 d. Payment made via EFS-Web.
5.  Any refund should be made by  check or  credit to Deposit Account No. 503994. 37 CFR 1.26(c). If payment is made by credit card, refund must be to credit card account.
6.  A copy of the patent to be reexamined having a double column format on one side of a separate paper is enclosed. 37 CFR 1.510(b)(4).
7.  CD-ROM or CD-R in duplicate, Computer Program (Appendix) or large table  
 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 (2 copies) or CD-R (2 copies); or  
ii.  paper  
c.  Statements verifying identity of above copies
9.  A copy of any disclaimer, certificate of correction or reexamination certificate issued in the patent is included.
10.  Reexamination of claim(s) 1-12, 14-16, 19-32, 38-44, 46-48, 51-64 is requested.
11.  A copy of every patent or printed publication relied upon is submitted herewith including a listing thereof on Form PTO/SB/08, PTO-1449, or equivalent.
12.  An English language translation of all necessary and pertinent non-English language patents and/or printed publications is included.

[Page 1 of 2]

This collection of information is required by 37 CFR 1.510. 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 18 minutes to complete, including gathering, preparing, and submitting the completed application form to the USPTO. Time will vary depending upon the individual case. Any comments on the amount of time you require to complete this form and/or suggestions for reducing this burden, should be sent to the Chief Information Officer, U.S. Patent and Trademark Office, U.S. Department of Commerce, P.O. Box 1450, Alexandria, VA 22313-1450. DO NOT SEND FEES OR COMPLETED FORMS TO THIS ADDRESS. **SEND TO: Mail Stop *Ex Parte* Reexam, Commissioner for Patents, P.O. Box 1450, Alexandria, VA 22313-1450.**

*If you need assistance in completing the form, call 1-800-PTO-9199 and select option 2.*

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13.  The attached detailed request includes at least the following items:
- A statement identifying each substantial new question of patentability based on prior patents and printed publications. 37 CFR 1.510(b)(1).
  - An identification of every claim for which reexamination is requested, and a detailed explanation of the pertinency and manner of applying the cited art to every claim for which reexamination is requested. 37 CFR 1.510(b)(2).
14.  A proposed amendment is included (only where the patent owner is the requester). 37 CFR 1.510(e).
15.  a. It is certified that a copy of this request (if filed by other than the patent owner) has been served in its entirety on the patent owner as provided in 37 CFR 1.33(c).  
The name and address of the party served and the date of service are:  
Banner & Witcoff, Ltd., Attorneys for client 001107, 1100 13th Street N.W., Suite 1200, Washington DC 20005-4051
- Date of Service: \_\_\_\_\_; or
- b. A duplicate copy is enclosed since service on patent owner was not possible. An explanation of the efforts made to serve patent owner **is attached**. See MPEP § 2220.

16. Correspondence Address: Direct all communication about the reexamination to:

The address associated with Customer Number:

52059

OR

Firm or  
Individual Name \_\_\_\_\_

Address

City

State

Zip

Country

Telephone

Email

17.  The patent is currently the subject of the following concurrent proceeding(s):

- a. Copending reissue Application No. \_\_\_\_\_
- b. Copending reexamination Control No. Concurrent requests in related patents 7824889 & 7915015
- c. Copending Interference No. \_\_\_\_\_
- d. Copending litigation styled:  
United States District Court for the Middle District of North Carolina Greensboro Division (Esoterix Genetic Labs, LLC, & The Johns Hopkins Univ. vs. Life Techs. Corp., Applied Biosystems, LLC, and Ion Torrent Systems, Inc., Case No. 12-1173 (Oct 31, 2012)

**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.**

/Ashita A. Doshi/

6/17/13

Authorized Signature

Date

Ashita Doshi

57,327

Typed/Printed Name

Registration No.

For Patent Owner Requester

For Third Party Requester

**IN THE UNITED STATES PATENT AND TRADEMARK OFFICE**

Patent No.: 6,440,706

Inventor: Bert Vogelstein et al.

Assignee: Johns Hopkins University

Issued: August 27, 2002

Application No.: 09/613,826

Filed: July 11, 2000

Control No.: To Be Assigned

Reexamination Filing Date: To Be Assigned

Art Unit: To Be Assigned

For: DIGITAL AMPLIFICATION

**CERTIFICATE OF SERVICE ON PATENT OWNER**  
**FOR EX PARTE REQUEST FOR REEXAMINATION**

I hereby certify that a copy of the Request for *Ex Parte* Reexamination by Third Party Requester filed on this date was served in its entirety on Patent Owner by First Class Mail, addressed to the following Correspondence Address of record for U.S. Patent No. 6,440,706:

Banner & Witcoff, Ltd.  
1100 13<sup>th</sup> Street N.W.  
Suite 1200  
Washington DC 20005-4051

Dated: June 17, 2013

Respectfully submitted,

By: /Elizabeth Morgan/  
Elizabeth Morgan  
Patent Paralegal  
Life Technologies Corporation  
2130 Woodward St., Bldg. 1  
Austin, TX 78744  
512-721-3690  
[Elizabeth.Morgan@lifetech.com](mailto:Elizabeth.Morgan@lifetech.com)

**Customer No. 52059**

## Electronic Patent Application Fee Transmittal

|                                             |                                   |
|---------------------------------------------|-----------------------------------|
| <b>Application Number:</b>                  |                                   |
| <b>Filing Date:</b>                         |                                   |
| <b>Title of Invention:</b>                  | Digital Amplification             |
| <b>First Named Inventor/Applicant Name:</b> | Bert Vogelstein                   |
| <b>Filer:</b>                               | Ashita Amu Doshi/Elizabeth Morgan |
| <b>Attorney Docket Number:</b>              | LT00831 REX                       |

Filed as Large Entity

### ex parte reexam Filing Fees

| Description                        | Fee Code | Quantity | Amount | Sub-Total in USD(\$) |
|------------------------------------|----------|----------|--------|----------------------|
| <b>Basic Filing:</b>               |          |          |        |                      |
| REQUEST FOR EX PARTE REEXAMINATION | 1812     | 1        | 12000  | 12000                |

**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(\$) |
|--------------------------|----------|----------|--------|----------------------|
| <b>Miscellaneous:</b>    |          |          |        |                      |
| <b>Total in USD (\$)</b> |          |          |        | <b>12000</b>         |

## Electronic Acknowledgement Receipt

|                                             |                                   |
|---------------------------------------------|-----------------------------------|
| <b>EFS ID:</b>                              | 16048211                          |
| <b>Application Number:</b>                  | 90012894                          |
| <b>International Application Number:</b>    |                                   |
| <b>Confirmation Number:</b>                 | 8442                              |
| <b>Title of Invention:</b>                  | Digital Amplification             |
| <b>First Named Inventor/Applicant Name:</b> | Bert Vogelstein                   |
| <b>Customer Number:</b>                     | 52059                             |
| <b>Filer:</b>                               | Ashita Amu Doshi/Elizabeth Morgan |
| <b>Filer Authorized By:</b>                 | Ashita Amu Doshi                  |
| <b>Attorney Docket Number:</b>              | LT00831 REX                       |
| <b>Receipt Date:</b>                        | 17-JUN-2013                       |
| <b>Filing Date:</b>                         |                                   |
| <b>Time Stamp:</b>                          | 16:32:23                          |
| <b>Application Type:</b>                    | Reexam (Third Party)              |

### Payment information:

|                                          |                 |
|------------------------------------------|-----------------|
| Submitted with Payment                   | yes             |
| Payment Type                             | Deposit Account |
| Payment was successfully received in RAM | \$12000         |
| RAM confirmation Number                  | 3829            |
| Deposit Account                          | 503994          |
| 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 1.20 (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)/<br>Message Digest      | Multi Part /.zip | Pages (if appl.) |
|---------------------|-----------------------------------------------------|-------------------------------------------------|------------------------------------------|------------------|------------------|
| 1                   | Copy of patent for which reexamination is requested | LT00831REX-Exhibit1-US6440706.pdf               | 1297861                                  | no               | 20               |
|                     |                                                     |                                                 | b4bd9b4fe944bd0a5ebf1a068389a676a76714f6 |                  |                  |
| <b>Warnings:</b>    |                                                     |                                                 |                                          |                  |                  |
| <b>Information:</b> |                                                     |                                                 |                                          |                  |                  |
| 2                   | Reexam - Affidavit/Decl/Exhibit Filed by 3rd Party  | LT00831REX-Exhibit3-US6440706-file-history.pdf  | 9057225                                  | no               | 207              |
|                     |                                                     |                                                 | 0b514d510082f06c05e4bfd2e202920a254bbd52 |                  |                  |
| <b>Warnings:</b>    |                                                     |                                                 |                                          |                  |                  |
| <b>Information:</b> |                                                     |                                                 |                                          |                  |                  |
| 3                   | Reexam - Affidavit/Decl/Exhibit Filed by 3rd Party  | LT00831REX-Exhibit4-60146792.pdf                | 1540417                                  | no               | 39               |
|                     |                                                     |                                                 | 912ebfcc55bebefb1d81e60bfe84d87a386bf21  |                  |                  |
| <b>Warnings:</b>    |                                                     |                                                 |                                          |                  |                  |
| <b>Information:</b> |                                                     |                                                 |                                          |                  |                  |
| 4                   | Reexam - Affidavit/Decl/Exhibit Filed by 3rd Party  | LT00831REX-Exhibit5-US13071105-file-history.pdf | 12475043                                 | no               | 189              |
|                     |                                                     |                                                 | 807ae0d932ca18c3d391b03adf1ad510f101f5dc |                  |                  |
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| <b>Information:</b> |                                                     |                                                 |                                          |                  |                  |
| 5                   | Reexam - Affidavit/Decl/Exhibit Filed by 3rd Party  | LT00831REX-Exhibit6-Kuppers-1993-4955.pdf       | 3796691                                  | no               | 14               |
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| 6                   | Reexam - Affidavit/Decl/Exhibit Filed by 3rd Party  | LT00831REX-Exhibit7-Wolf-1996-3418.pdf          | 5486030                                  | no               | 12               |
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| <b>Information:</b> |                                                     |                                                 |                                          |                  |                  |
| 7                   | Reexam - Affidavit/Decl/Exhibit Filed by 3rd Party  | LT00831REX-Exhibit-PA1-Li-1998.pdf              | 474790                                   | no               | 5                |
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| <b>Information:</b> |                                                     |                                                 |                                          |                  |                  |
| 8                   | Reexam - Affidavit/Decl/Exhibit Filed by 3rd Party  | LT00831REX-Exhibit-PA2-Zhang-1992.pdf           | 8167591                                  | no               | 6                |
|                     |                                                     |                                                 | b686a0f3cacda90fc6a0423f9c2c3fd43776c0d4 |                  |                  |
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|---------------------|-----------------------|-----------------------------------------------|-------------------------------------------------------|----|----|
| <b>Information:</b> |                       |                                               |                                                       |    |    |
| 9                   | Non Patent Literature | LT00831REX-Exhibit-PA3-Jeffreys-1988.pdf      | 15549635<br>47789347f3441ca6da0508c904b2fd644d8acdf7  | no | 20 |
| <b>Warnings:</b>    |                       |                                               |                                                       |    |    |
| <b>Information:</b> |                       |                                               |                                                       |    |    |
| 10                  | Non Patent Literature | LT00831REX-Exhibit-PA4-Kalinina-1997-1999.pdf | 8517931<br>9f3eab3092041cd3fbc7b13f8620af7091b90565   | no | 7  |
| <b>Warnings:</b>    |                       |                                               |                                                       |    |    |
| <b>Information:</b> |                       |                                               |                                                       |    |    |
| 11                  | Non Patent Literature | LT00831REX-Exhibit-PA5-Chou-1992-1717.pdf     | 874158<br>80b63820ab73ed4ba8f3c7c884f0e052ef9e9d40    | no | 8  |
| <b>Warnings:</b>    |                       |                                               |                                                       |    |    |
| <b>Information:</b> |                       |                                               |                                                       |    |    |
| 12                  | Non Patent Literature | LT00831REX-Exhibit-PA6-Burg-1989-1787.pdf     | 11113342<br>dbd934ddffa779f6a97e58157d27c1754e9a3788  | no | 7  |
| <b>Warnings:</b>    |                       |                                               |                                                       |    |    |
| <b>Information:</b> |                       |                                               |                                                       |    |    |
| 13                  | Non Patent Literature | LT00831REX-Exhibit-PA7-Trumper-1993-3097.pdf  | 24684508<br>9c4f7a630f39e0f53dd6e4754a5e5b87e77fd8f8  | no | 20 |
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| <b>Information:</b> |                       |                                               |                                                       |    |    |
| 14                  | Non Patent Literature | LT00831REX-Exhibit-PA8-Kanzler-1996-3429.pdf  | 12057722<br>b457da6c4c12302140f928b1f6c8757a9dca4dbd8 | no | 9  |
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| <b>Information:</b> |                       |                                               |                                                       |    |    |
| 15                  | Non Patent Literature | LT00831REX-Exhibit-PA9-Gravel-1998-2866.pdf   | 4239688<br>210edc8f3fc40bc22f66c78a942d5775c2b01bb    | no | 10 |
| <b>Warnings:</b>    |                       |                                               |                                                       |    |    |
| <b>Information:</b> |                       |                                               |                                                       |    |    |
| 16                  | Non Patent Literature | LT00831REX-Exhibit-PA10-Marcucci-1998-790.pdf | 7432863<br>2b58de8f9756530ad6286d1103f624ba066ae607   | no | 5  |
| <b>Warnings:</b>    |                       |                                               |                                                       |    |    |
| <b>Information:</b> |                       |                                               |                                                       |    |    |
| 17                  | Non Patent Literature | LT00831REX-Exhibit-PA11-Flint-1997-2469.pdf   | 381469<br>ecfa37f2c69afa51ec12a478b597f3e412048ccd    | no | 9  |
| <b>Warnings:</b>    |                       |                                               |                                                       |    |    |

| <b>Information:</b>                 |                                                       |                                                           |                                                     |    |     |
|-------------------------------------|-------------------------------------------------------|-----------------------------------------------------------|-----------------------------------------------------|----|-----|
| 18                                  | Non Patent Literature                                 | LT00831REX-Exhibit-PA12-Ponten-1997-45.pdf                | 1784465<br>9fcfce5e11f151e78c5798cd494ab789af37acd5 | no | 12  |
| <b>Warnings:</b>                    |                                                       |                                                           |                                                     |    |     |
| <b>Information:</b>                 |                                                       |                                                           |                                                     |    |     |
| 19                                  | Reexam - Affidavit/Decl/Exhibit Filed by 3rd Party    | LT00831REX-Exhibit8-Mandahl-1996-632.pdf                  | 5469597<br>da64853c602b15ac80e4c1d9a1f53027ab33c2d8 | no | 5   |
| <b>Warnings:</b>                    |                                                       |                                                           |                                                     |    |     |
| <b>Information:</b>                 |                                                       |                                                           |                                                     |    |     |
| 20                                  | Reexam - Info Disclosure Statement Filed by 3rd Party | LT00831REX-Exhibit2-SB08.pdf                              | 90873<br>ad98141d0c4284a7e6bb97726677ef456e8780cb   | no | 3   |
| <b>Warnings:</b>                    |                                                       |                                                           |                                                     |    |     |
| <b>Information:</b>                 |                                                       |                                                           |                                                     |    |     |
| 21                                  | Receipt of Orig. Ex Parte Request by Third Party      | LT00831REX-reexam-request-6-17-13.pdf                     | 616962<br>aada374bf488e7d4e4cbebe43e5b147ca6c97174  | no | 168 |
| <b>Warnings:</b>                    |                                                       |                                                           |                                                     |    |     |
| <b>Information:</b>                 |                                                       |                                                           |                                                     |    |     |
| 22                                  | Transmittal of New Application                        | LT00831REX-reexam-request-transmittal-6-17-13.pdf         | 243291<br>c2493d3b4aff11ffe6e3ebf05a65d54208f1507c  | no | 2   |
| <b>Warnings:</b>                    |                                                       |                                                           |                                                     |    |     |
| <b>Information:</b>                 |                                                       |                                                           |                                                     |    |     |
| 23                                  | Reexam Certificate of Service                         | LT00831REX-certificate-service-reexam-request-6-17-13.pdf | 87905<br>4277491547b034b56252f5d5a177c0457d529d4c   | no | 1   |
| <b>Warnings:</b>                    |                                                       |                                                           |                                                     |    |     |
| <b>Information:</b>                 |                                                       |                                                           |                                                     |    |     |
| 24                                  | Fee Worksheet (SB06)                                  | fee-info.pdf                                              | 29588<br>d407d9cf71f14d4f6f5db4f9fdd1181aefde2647   | no | 2   |
| <b>Warnings:</b>                    |                                                       |                                                           |                                                     |    |     |
| <b>Information:</b>                 |                                                       |                                                           |                                                     |    |     |
| <b>Total Files Size (in bytes):</b> |                                                       |                                                           | 135469645                                           |    |     |

**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.**

# EXHIBIT 1



US006440706B1

(12) **United States Patent**  
**Vogelstein et al.**

(10) **Patent No.:** **US 6,440,706 B1**  
(45) **Date of Patent:** **Aug. 27, 2002**

(54) **DIGITAL AMPLIFICATION**

(75) Inventors: **Bert Vogelstein**, Baltimore; **Kenneth W. Kinzler**, BelAir, both of MD (US)

(73) Assignee: **Johns Hopkins University**, Baltimore, MD (US)

(\* ) Notice: Subject to any disclaimer, the term of this patent is extended or adjusted under 35 U.S.C. 154(b) by 0 days.

(21) Appl. No.: **09/613,826**

(22) Filed: **Jul. 11, 2000**

**Related U.S. Application Data**

(60) Provisional application No. 60/146,792, filed on Aug. 2, 1999.

(51) **Int. Cl.**<sup>7</sup> ..... **C12P 19/34**; C12Q 1/68; C07H 21/02; C07H 21/04; C07H 19/00

(52) **U.S. Cl.** ..... **435/91.2**; 435/6; 435/7.1; 435/91.1; 536/22.1; 536/23.1; 536/24.3; 536/24.31; 536/24.32; 536/24.33

(58) **Field of Search** ..... 435/6, 7.1, 91.1, 435/91.2; 536/22.1, 23.1, 24.3, 24.31, 24.32, 24.33

(56) **References Cited**

**U.S. PATENT DOCUMENTS**

|           |   |          |                 |       |
|-----------|---|----------|-----------------|-------|
| 5,670,325 | A | 9/1997   | Lapidus et al.  |       |
| 5,804,383 | A | 9/1998   | Gruenert et al. |       |
| 5,858,663 | A | 1/1999   | Nisson et al.   |       |
| 5,925,517 | A | * 7/1999 | Tyagi et al.    | 435/6 |
| 5,928,870 | A | 7/1999   | Lapidus et al.  |       |
| 6,020,137 | A | 2/2000   | Lapidus et al.  |       |
| 6,037,130 | A | * 3/2000 | Tyagi et al.    | 435/6 |
| 6,143,496 | A | 11/2000  | Brown et al.    |       |

**FOREIGN PATENT DOCUMENTS**

|    |             |   |        |
|----|-------------|---|--------|
| EP | 0643140     | A | 3/1995 |
| WO | WO 95/13399 |   | 5/1995 |
| WO | WO 99/13113 |   | 3/1999 |

**OTHER PUBLICATIONS**

Newton Essential PCR pp. 51–52 1995.\*  
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*Primary Examiner*—Jeffrey Siew  
(74) *Attorney, Agent, or Firm*—Banner & Witcoff, Ltd.

(57) **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 mutations. The process provides a reliable and quantitative measure of the proportion of variant sequences within a DNA sample.

**64 Claims, 7 Drawing Sheets**



FIG. 1A

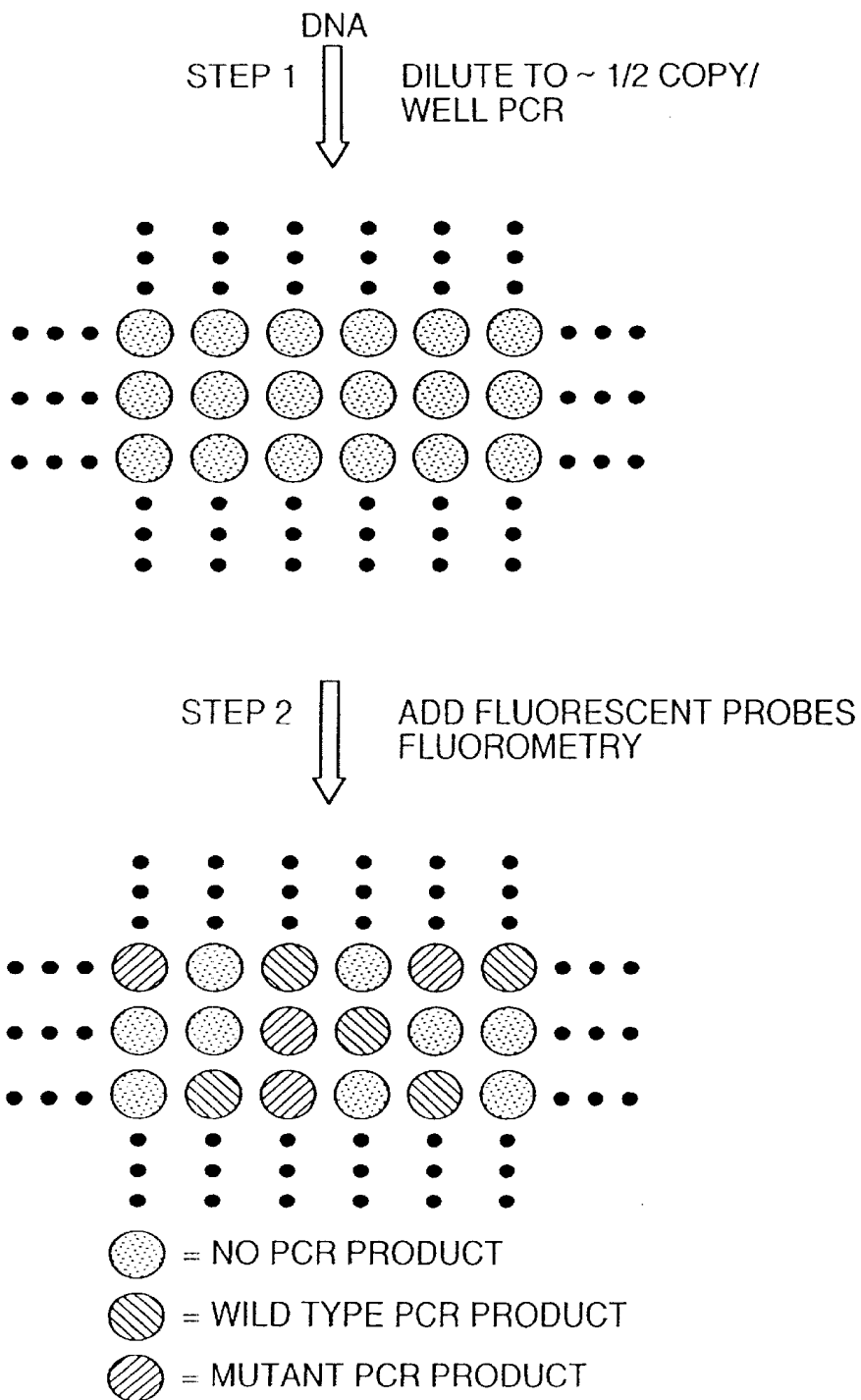


FIG. 1B

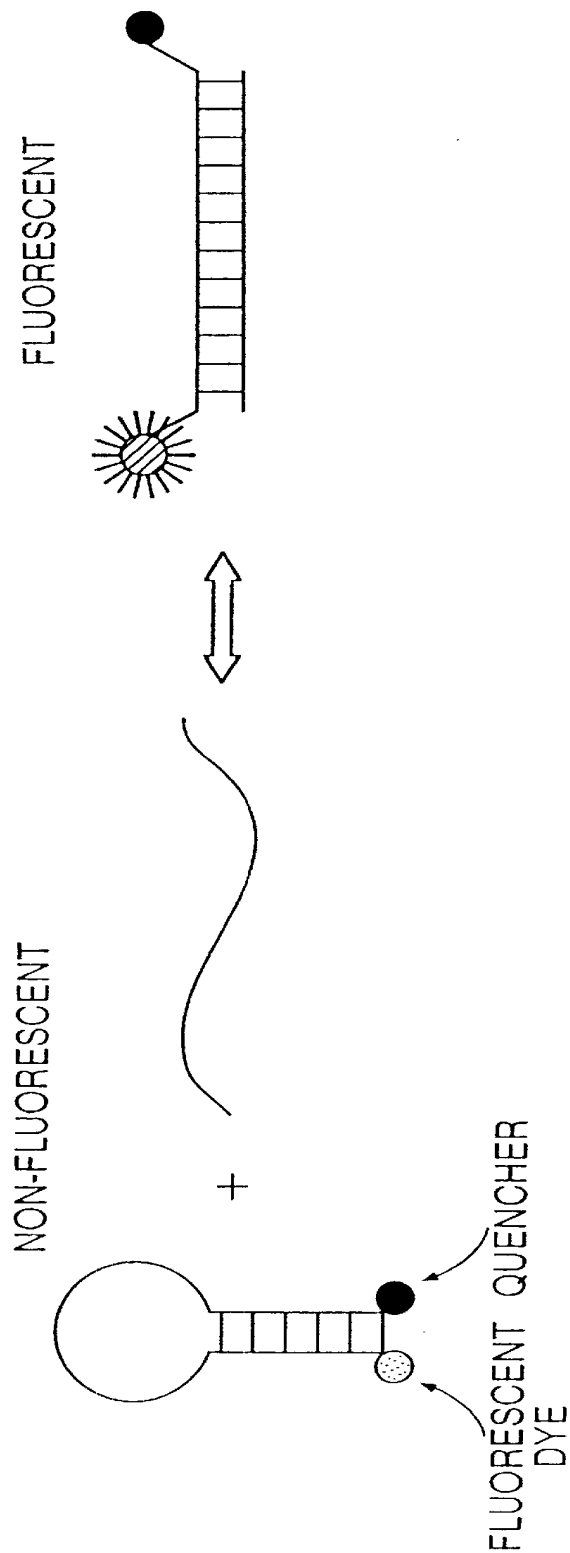


FIG. 1C

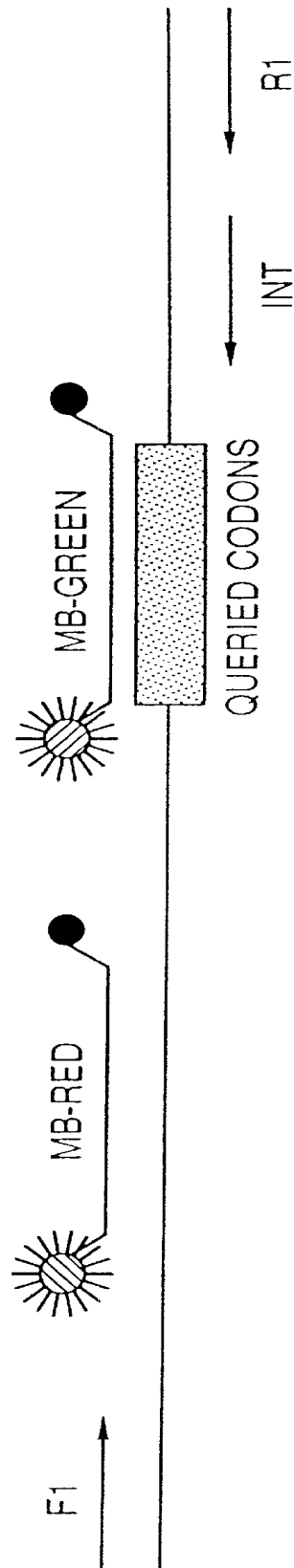


FIG. 2

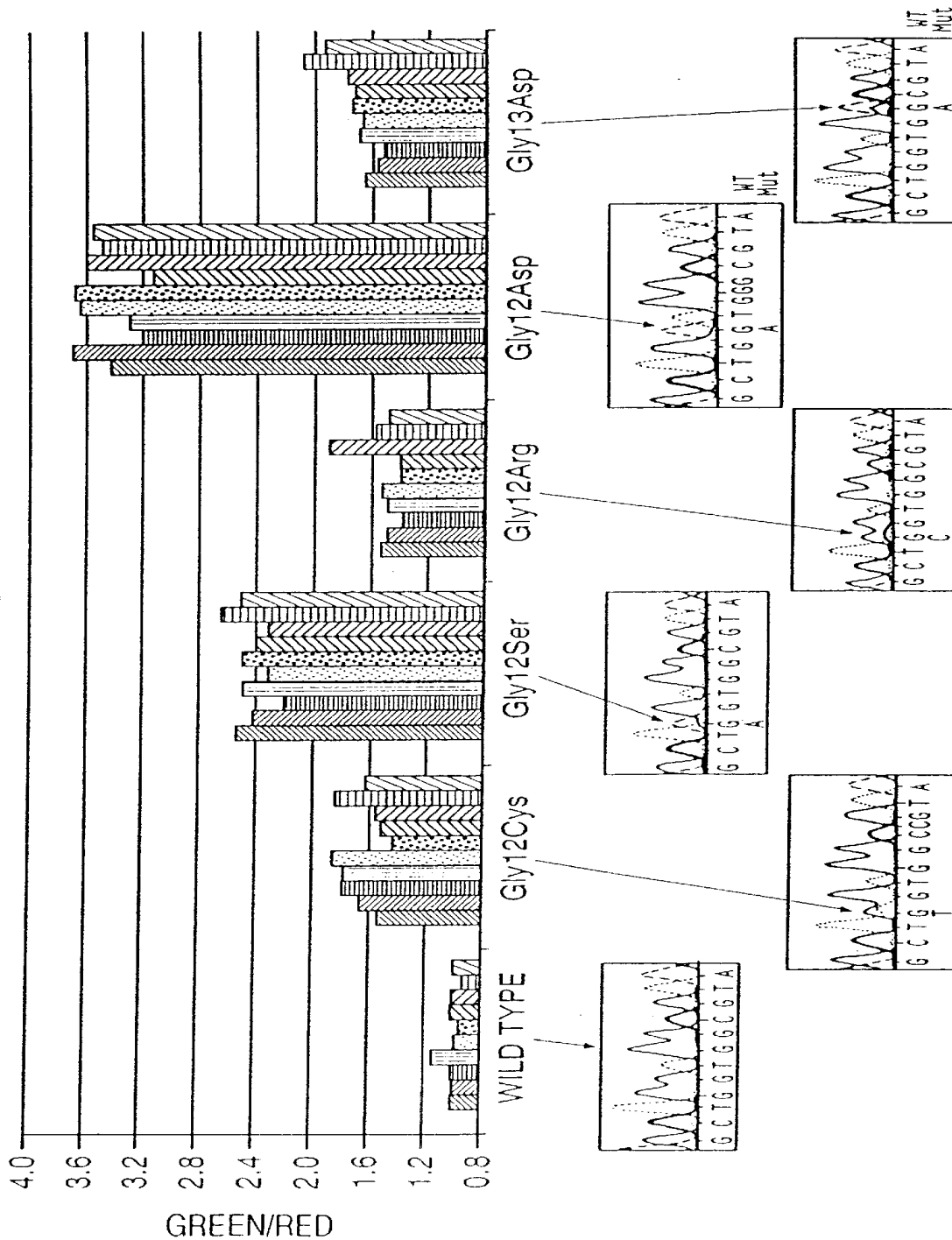
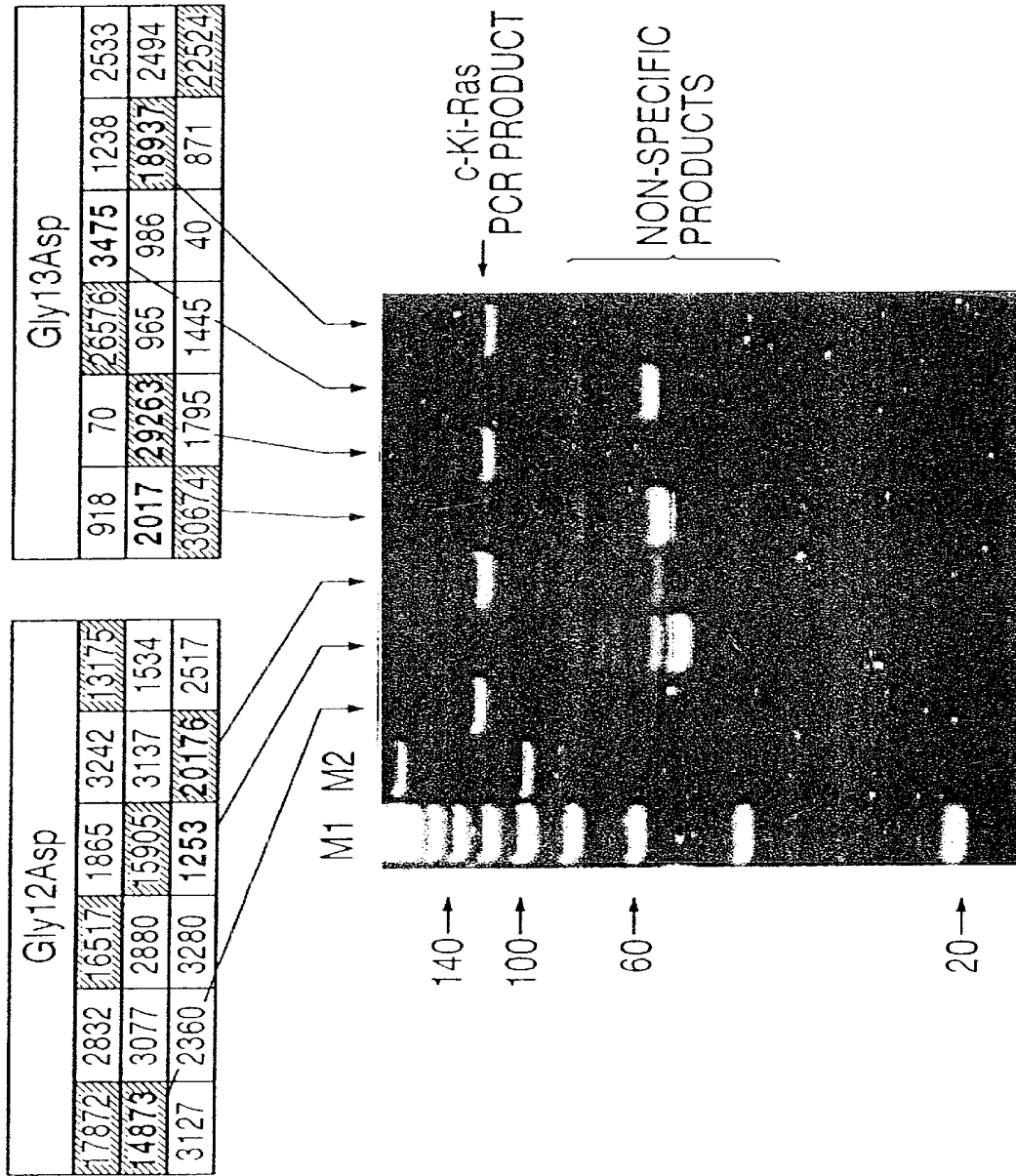


FIG. 3



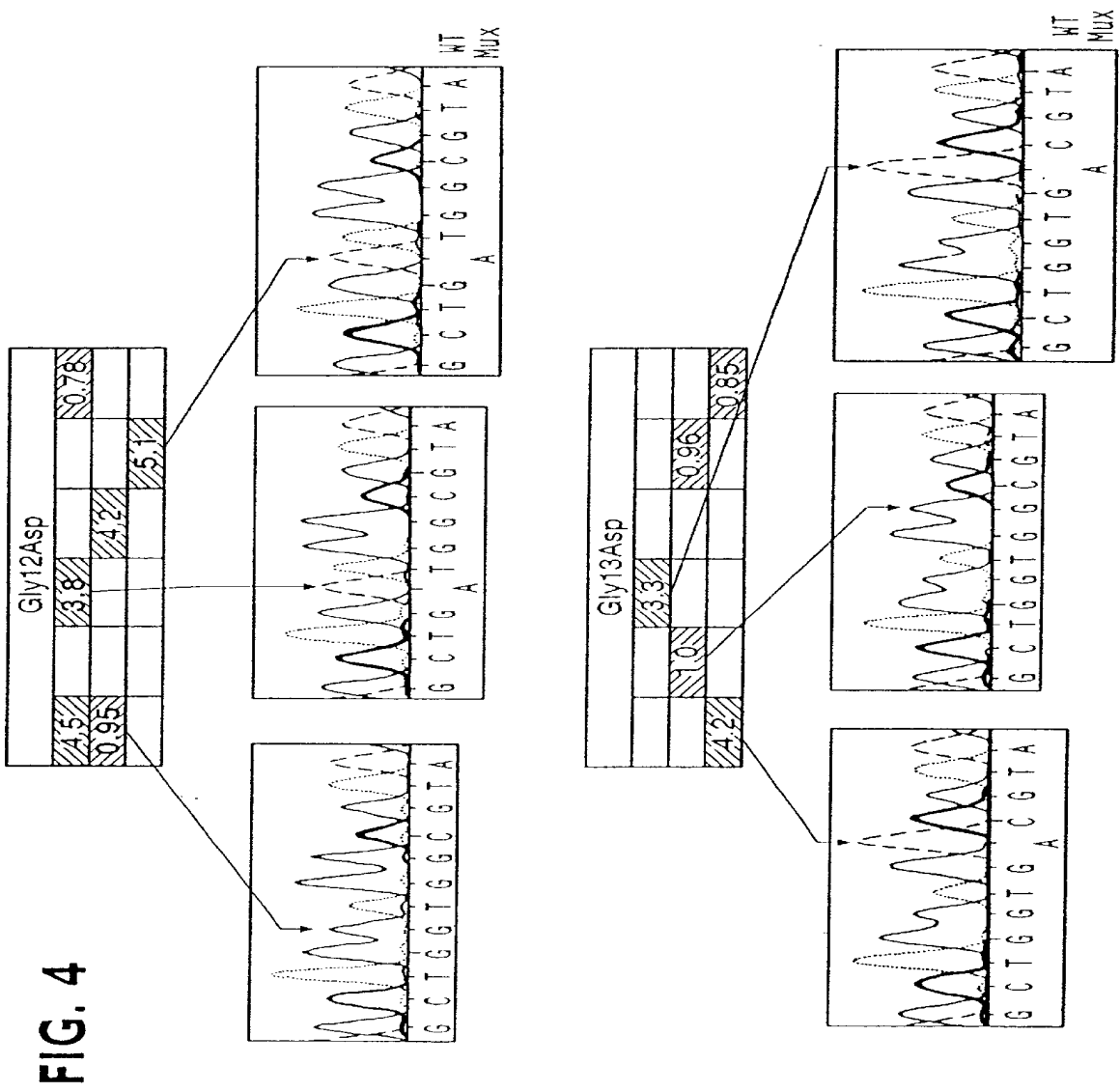
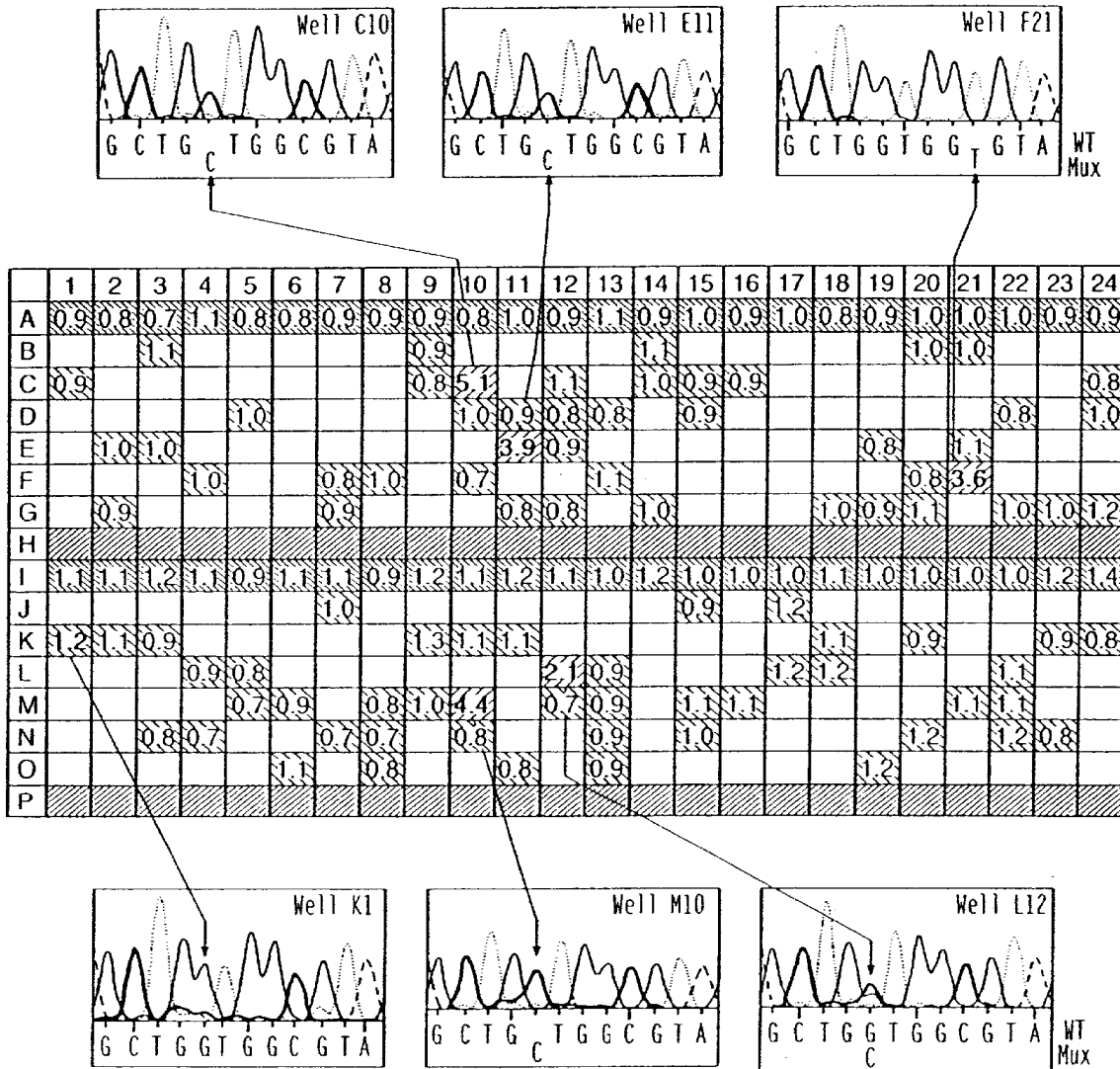


FIG. 5



**DIGITAL AMPLIFICATION**

This application claims the benefit of U.S. Ser. No. 60/146,792, filed Aug. 2, 1999.

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.

**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 (1), and may also play a role in aging (2,3), 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 (4), stool (5,6), and sputum (7,8) 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 (9-11). The detection of residual disease in lymph nodes or surgical margins may be useful in predicting which patients might benefit most from further therapy (12-14). 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 (15-17).

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% (18,19). 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 (20-22). 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 (23-28). Other innovative approaches for the detection of somatic mutations have been reviewed (29-32). 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. 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 and 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**

FIGS. 1A, 1B, 1C. Schematic of experimental design. (A) 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. (B) 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. (C) 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



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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 K1 (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

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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 <0.3%, i.e., in control experiments with DNA from normal cells, none of 340 wells containing PCR products exhibited RED/GREEN ratios >3.0. Any individual mutation (such as a G- to C-transversion at the second position of codon 12 of c-Ki-ras) is expected to occur in <1 in 50 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 (55)).

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.

TABLE 1

## Potential Applications of Dig-PCR

| 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                                   |

TABLE 1-continued

| Potential Applications of Dig-PCR |                                                    |                             |                                  |
|-----------------------------------|----------------------------------------------------|-----------------------------|----------------------------------|
| Application                       | Example                                            | Probe 1 Detects:            | Probe 2 Detects:                 |
| Allelic imbalance                 | Quantitative analysis with non-polymorphic markers | marker from test chromosome | marker from reference chromosome |

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 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 (Marsh Biomedical Products, Rochester, N.Y.). The composition of the reactions was: 67 mM Tris, pH 8.8, 16.6 mM NH<sub>4</sub>SO<sub>4</sub>, 6.7 mM MgCl<sub>2</sub>, 10 mM β-mercaptoethanol, 1 mM dATP, 1 mM dCTP, 1 mM dGTP, 1 mM TTP, 6% DMSO, 1 uM

10 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 ~1.5 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<sub>4</sub>SO<sub>4</sub>, 6.7 mM MgCl<sub>2</sub>, 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. The 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; 94° for one minute; and 60° for five minutes. The plates were then incubated at room temperature for ten to sixty minutes and fluorescence measured as described above. 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 above in Example 1). 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-CACGGCCTGCTGAAAATGACTGCGTG-  
Dabcyl-3' (SEQ ID NO: 4);

MB-GREEN: 5'-Fluorescein-  
CACGGGAGCTGGTGGCGTAGCGTG-Dabcyl-3'  
(SEQ ID NO: 5). Molecular Beacons (**33,34**) were synthesized by Midland Scientific and other oligonucleotides were synthesized by Gene Link (Thomwood, N.Y.). All were dissolved at 50  $\mu$ M 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'-CATTATTTTATTATAAGGCCTGC-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 (**31, 33-40**). We chose to explore the utility of one such technology, involving Molecular Beacons (MB), for this purpose (**33,34**). 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<sup>th</sup> 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 (**41**). 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 its hybridization to the MB probe (**33,34**). 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 (**44,45**) 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 raise the background fluorescence and cost of the assay. 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 (T<sub>m</sub>) 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 T<sub>m</sub> 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 genome equivalents of DNA were added to 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 were illustrated 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 of 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  $\pm$  s.d. of 47,000  $\pm$  18,000 SFU) while the other 186 wells did not (2600  $\pm$  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. 5). 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  $\sim$ 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).

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## SEQUENCE LISTING

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What is claimed is:

1. A method for determining the ratio of a selected genetic sequence in a population of genetic sequences, 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 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 biological sample.

2. The method of claim 1 wherein the step of diluting is performed until at least one-tenth 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 for the step of analyzing to determine the presence of the selected genetic sequence.

3. 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.

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 10 nucleic acid template molecules containing the reference genetic sequence.

5. 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 the reference genetic sequence.

6. The method of claim 1 wherein the biological sample is cell-free.

7. The method of claim 1 wherein the number of assay samples within the set is greater than 10.

8. The method of claim 1 wherein the number of assay samples within the set is greater than 50.

9. The method of claim 1 wherein the number of assay samples within the set is greater than 100.

10. The method of claim 1 wherein the number of assay samples within the set is greater than 500.

11. The method of claim 1 wherein the number of assay samples within the set is greater than 1000.

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

13. 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.

14. The method of claim 1 wherein the step of analyzing employs gel electrophoresis.

15. The method of claim 1 wherein the step of analyzing employs hybridization to at least one nucleic acid probe.

16. The method of claim 1 wherein the step of analyzing employs hybridization to at least two nucleic acid probe.

17. The method of claim 13 wherein two molecular beacon probes are used, each having a different photoluminescent dye.

18. The method of claim 13 wherein the molecular beacon probe detects a wild-type selected genetic sequence better than a mutant selected genetic sequence.

19. The method of claim 1 wherein the step of amplifying employs a single pair of primers.

20. The method of claim 1 wherein the step of amplifying employs a polymerase which is activated only after heating.

21. The method of claim 1 wherein the step of amplifying employs at least 40 cycles of heating and cooling.

22. The method of claim 1 wherein the step of amplifying employs at least 50 cycles of heating and cooling.

23. The method of claim 1 wherein the step of amplifying employs at least 60 cycles of heating and cooling.

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



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25. 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.

26. The method of claim 1 wherein the selected genetic sequence is a translocated allele.

27. The method of claim 1 wherein the selected genetic sequence is a wild-type allele.

28. The method of claim 1 wherein the selected genetic sequence is within an amplicon which is amplified during neoplastic development.

29. The method of claim 1 wherein the selected genetic sequence is a rare exon sequence.

30. The method of claim 1 wherein the nucleic acid template molecules comprise cDNA of RNA transcripts and the selected genetic sequence is present on a cDNA of a first transcript and the reference genetic sequence is present on a cDNA of a second transcript.

31. The method of claim 1 wherein the selected genetic sequence comprises a first mutation and the reference genetic sequence comprises a second mutation.

32. The method of claim 1 wherein the selected genetic sequence and the reference genetic sequence are on distinct chromosomes.

33. A molecular beacon probe comprising:

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, wherein the loop consists of 16 base pairs, wherein the loop has a  $T_m$  of 50–51° C. and the stem consists of 4 base pairs having a sequence 5'-CACG-3'.

34. The probe of claim 33 wherein the molecular beacon probe detects a wild-type selected genetic sequence better than a mutant selected genetic sequence.

35. The probe of claim 33 wherein the molecular beacon probe detects a mutant genetic sequence better than a wild-type genetic sequence.

36. A molecular beacon probe comprising:

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, wherein the loop consists of 19–20 base pairs, wherein the loop has a  $T_m$  of 54–56° C. and the stem consists of 4 base pairs having a sequence 5'-CACG-3'.

37. A pair of molecular beacon probes comprising:

a first molecular beacon probe which is an oligonucleotide with a stem-loop structure having a first photoluminescent dye at one of the 5' or 3' ends and a quenching agent at the opposite 5' or 3' end, wherein the loop consists of 16 base pairs having a  $T_m$  of 50–51° C. and the stem consists of 4 base pairs having a sequence 5'-CACG-3'; and

a second molecular beacon probe which is an oligonucleotide with a stem-loop structure having a second photoluminescent dye at one of the 5' or 3' ends and a quenching agent at the opposite 5' or 3' end, wherein the loop consists of 19–20 base pairs having a  $T_m$  of 54–56° C. and the stem consists of 4 base pairs having a sequence 5'-CACG-3';

wherein the first and the second photoluminescent dyes are distinct.

38. A method for determining the ratio of a selected genetic sequence in a population of genetic sequences, comprising the steps of:

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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;

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 biological sample.

39. The method of claim 38 wherein the number of assay samples within the set is greater than 10.

40. The method of claim 38 wherein the number of assay samples within the set is greater than 50.

41. The method of claim 38 wherein the number of assay samples within the set is greater than 100.

42. The method of claim 38 wherein the number of assay samples within the set is greater than 500.

43. The method of claim 38 wherein the number of assay samples within the set is greater than 1000.

44. The method of claim 38 wherein the step of amplifying and the step of analyzing are performed on assay samples in the same receptacle.

45. The method of claim 38 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.

46. The method of claim 38 wherein the step of analyzing employs gel electrophoresis.

47. The method of claim 38 wherein the step of analyzing employs hybridization to at least one nucleic acid probe.

48. The method of claim 38 wherein the step of analyzing employs hybridization to at least two nucleic acid probe.

49. The method of claim 45 wherein two molecular beacon probes are used, each having a different photoluminescent dye.

50. The method of claim 45 wherein the molecular beacon probe detects a wild-type selected genetic sequence better than a mutant selected genetic sequence.

51. The method of claim 38 wherein the step of amplifying employs a single pair of primers.

52. The method of claim 38 wherein the step of amplifying employs a polymerase which is activated only after heating.

53. The method of claim 38 wherein the step of amplifying employs at least 40 cycles of heating and cooling.

54. The method of claim 38 wherein the step of amplifying employs at least 50 cycles of heating and cooling.

55. The method of claim 38 wherein the step of amplifying employs at least 60 cycles of heating and cooling.

56. The method of claim 38 wherein the template molecules are obtained from a body sample selected from the group consisting of stool, blood, and lymph nodes.

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**57.** The method of claim **38** wherein the template molecules are obtained from a body sample of a leukemia or lymphoma patient who has received anti-cancer therapy, said body sample being selected from the group consisting of blood and bone marrow.

**58.** The method of claim **38** wherein the selected genetic sequence is a translocated allele.

**59.** The method of claim **38** wherein the selected genetic sequence is a wild-type allele.

**60.** The method of claim **38** wherein the selected genetic sequence is within an amplicon which is amplified during neoplastic development.

**61.** The method of claim **38** wherein the selected genetic sequence is a rare exon sequence.

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**62.** The method of claim **38** wherein the nucleic acid template molecules comprise cDNA of RNA transcripts and the selected genetic sequence is present on a cDNA of a first transcript and the reference genetic sequence is present on a cDNA of a second transcript.

**63.** The method of claim **38** wherein the selected genetic sequence comprises a first mutation and the reference genetic sequence comprises a second mutation.

**64.** The method of claim **38** wherein the selected genetic sequence and the reference genetic sequence are on distinct chromosomes.

\* \* \* \* \*

# EXHIBIT 3

|     |     |          |                      |
|-----|-----|----------|----------------------|
| 435 | 912 | Subclass | ISSUE CLASSIFICATION |
|     |     | Class    |                      |

PATENT NUMBER  
**6440705**

**U.S. UTILITY Patent Application**

50  
52551

SCANNED *RW* O.I.P.E. *GU* PATENT DATE **AUG 27 2002**  
D.A. *CK*

| APPLICATION NO. | CONT/PRIOR | CLASS | SUBCLASS | ART UNIT | EXAMINER |
|-----------------|------------|-------|----------|----------|----------|
| 09/613826       | D          | 435   | 8 912    | 1638     | 74907    |

APPLICANTS: Bart Vogelstein  
Kenneth Kinzler

Digital amplification

PTO-2040  
12/89

| ISSUING CLASSIFICATION       |          |                    |                                   |      |      |       |       |       |  |
|------------------------------|----------|--------------------|-----------------------------------|------|------|-------|-------|-------|--|
| ORIGINAL                     |          | CROSS REFERENCE(S) |                                   |      |      |       |       |       |  |
| CLASS                        | SUBCLASS | CLASS              | SUBCLASS (ONE SUBCLASS PER BLOCK) |      |      |       |       |       |  |
| 435                          | 912      | 435                | 6                                 | 71   | 911  |       |       |       |  |
| INTERNATIONAL CLASSIFICATION |          | 536                | 221                               | 23.1 | 24.3 | 24.31 | 24.32 | 24.33 |  |
| C12P                         | 19/34    |                    |                                   |      |      |       |       |       |  |
| C12Q                         | 1/68     |                    |                                   |      |      |       |       |       |  |
| C07H                         | 21/02    |                    |                                   |      |      |       |       |       |  |
| C07H                         | 21/04    |                    |                                   |      |      |       |       |       |  |
| C07H                         | 19/00    |                    |                                   |      |      |       |       |       |  |

|                                                                                                                                                                                                                                                                                                                                                                                         |                                                                                      |  |  |                                                                                |  |
|-----------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------|--------------------------------------------------------------------------------------|--|--|--------------------------------------------------------------------------------|--|
| <input type="checkbox"/> <b>TERMINAL DISCLAIMER</b><br><br><input type="checkbox"/> The term of this patent subsequent to _____ (date) has been disclaimed.<br><br><input type="checkbox"/> The term of this patent shall not extend beyond the expiration date of U.S. Patent No. _____<br><br><input type="checkbox"/> The terminal _____ months of this patent have been disclaimed. | <b>DRAWINGS</b><br>Sheets Drwg. <b>7</b> Figs. Drwg. <b>7</b> Print Fig. <b>None</b> |  |  | <b>CLAIMS ALLOWED</b><br>Total Claims <b>64</b> Print Claim for O.G. <b>1</b>  |  |
|                                                                                                                                                                                                                                                                                                                                                                                         | NOTICE OF ALLOWANCE MAILED<br><b>March 26, 2002</b>                                  |  |  | <b>ISSUE FEE</b> <i>W</i><br>Amount Due <b>640.00</b> Date Paid <b>5-21-02</b> |  |
|                                                                                                                                                                                                                                                                                                                                                                                         | Jeffrey Siew <b>3/19/02</b><br><small>(Primary Examiner)</small>                     |  |  | <b>ISSUE BATCH NUMBER</b><br><b>9893</b>                                       |  |

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PATENT APPLICATION



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INITIALS

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| SMD<br>reference<br>target<br>single molecule<br>genom. eg. unclat<br>molecule bases<br>loop hump in<br>stem<br>tm<br>melting temperature | ↓       | ↓     |
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ISSUE SLIP STAPLE AREA (for additional cross references)

| POSITION                  | INITIALS | ID NO. | DATE     |
|---------------------------|----------|--------|----------|
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| O.I.P.E. CLASSIFIER       | PH       |        | 7/22     |
| FORMALITY REVIEW          | BS       | 551    | 8-24-00  |
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|-----------------------------|---------------------------|--------------|------------------------|---------------------------------------|
| SERIAL NUMBER<br>09/813,826 | FILING DATE<br>07/11/2000 | CLASS<br>435 | GROUP ART UNIT<br>1637 | ATTORNEY<br>DOCKET NO.<br>01107.00031 |
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APPLICANTS

Bert Vogelstein, Baltimore, MD;  
Kenneth W. Kinzler, BelAir, MD;

\*\* CONTINUING DATA \*\*\*\*\*  
THIS APPLN CLAIMS BENEFIT OF 60/146,792 08/02/1999

\*\* FOREIGN APPLICATIONS \*\*\*\*\*

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\*\* 08/29/2000

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| Foreign Priority claimed<br>35 USC 119 (a-d) conditions met | <input type="checkbox"/> yes <input checked="" type="checkbox"/> no | STATE OR<br>COUNTRY | SHEETS<br>DRAWING | TOTAL<br>CLAIMS | INDEPENDENT<br>CLAIMS |
| Verified and Acknowledged                                   | Allowance<br>Examiner's Signature Initials                          | MD                  | 7                 | 64              | 5                     |

ADDRESS  
22907  
BANNER & WITCOFF  
1001 G STREET N W  
SUITE 1100  
WASHINGTON, DC  
20001

TITLE  
Digital application

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## DIGITAL AMPLIFICATION

### ABSTRACT

5 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 mutations. The process provides a reliable and quantitative  
10 measure of the proportion of variant sequences within a DNA sample.

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## DIGITAL AMPLIFICATION

This application claims the benefit of U.S. Serial No. 60/146,792, filed August 2, 1999.

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.

### 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 (1), and may also play a role in aging (2,3), 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 (4), stool (5,6), and sputum (7,8) 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 (9-11). The detection of residual disease in lymph nodes or surgical margins



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may be useful in predicting which patients might benefit most from further therapy (12-14). 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 (15-17).

5  
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% (18,19). Mutant-specific oligonucleotides can sometimes be used to detect  
10 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 (20-22). 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  
15 quantitate the fraction of mutant molecules in the starting population with these techniques (23-28). Other innovative approaches for the detection of somatic mutations have been reviewed (29-32). A general problem with these methods is that it is difficult or impossible to independently confirm the existence of any mutations that are identified.

20 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**

25 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.

30 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





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).

10 *Sub C2* 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.

15  
20 *Sub C3* 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 288 wells contained diluted DNA from the stool sample prepared by alkaline extraction. (Rubeck et al., 1998, *BioTechniques* 25:588-592.)  
25 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.

**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 <0.3%, *i.e.*, in control experiments with DNA from normal cells, none of 340 wells containing PCR products exhibited RED/GREEN ratios >3.0. Any individual mutation (such as a G- to C- transversion at the second position of codon 12 of *c-Ki-ras*) is expected to occur in <1 in 50 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

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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 (55)),

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





CONFIDENTIAL

Table

Table 1. Potential Applications of Dig-PCR

| 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 from test chromosome    | marker from reference chromosome                  |

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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

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TaqMan™ (dual-labeled fluorogenic) probes (Perkin Elmer Corp./Applied Biosystems, Foster City, Calif), pyrene-labeled probes, and other biochemical assays.

5 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

10 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 (Marsh Biomedical Products, Rochester, NY). The composition of the reactions was: 67 mM Tris, pH 8.8, 16.6 mM NH<sub>4</sub>SO<sub>4</sub>, 6.7 mM MgCl<sub>2</sub>, 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 ~1.5 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 20 25 36 hours before fluorescence analysis.

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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<sub>4</sub>SO<sub>4</sub>, 6.7 mM MgCl<sub>2</sub>, 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. The 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; 94° for one minute; and 60° for five minutes. The plates were then incubated at room temperature for ten to sixty minutes and fluorescence measured as described above. 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 above in Example 1). 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

30 *Sub*  
*0.3* Oligonucleotides and DNA sequencing. Primer F1:  
 5'-CATGTTCTAAATATAGTCACATTTTCA-3'; Primer R1:  
 5'-TCTGAATTAGCTGTATCGTCAAGG-3'; Primer INT:

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5'-TAGCTGTATCGTCAAGGCAC-3'; MB-RED:  
5'-Cy3-CACGGGCTGCTGAAAATGACTGCGTG-Dabcyl-3';  
M B - G R E E N :  
5'-Fluorescein-CACGGGAGCTGGTGGCGTAGCGTG-Dabcyl-3'.

5 Molecular Beacons (33,34) were synthesized by Midland Scientific and other oligonucleotides were synthesized by Gene Link (Thornwood, NY). 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'-CATTATTTTATTATAAGGCCTGC-3'. 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 (31, 33-40). We chose to explore the utility of one such technology, involving Molecular Beacons (MB), for this purpose (33,34). MB probes are oligonucleotides with stem-loop structures that contain a

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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<sup>th</sup> 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 (41). 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.

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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 its hybridization to the MB probe (33,34). 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.

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**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

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5 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 (44,45) 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.

15 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 raise the background

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5 fluorescence and cost of the assay. 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, 10 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 15 between closely related sequences, with a shorter region of homology facilitating such discrimination.

20 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 genome equivalents of DNA were added to 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 25 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 30 allele exhibited a ratio of 3.4 (Gly12Asp). These data suggested that wells

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

5            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  
10            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  
15            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  
20            addition of the MB probes.

#### EXAMPLE 5

15            Analysis of DNA from tumor cells. The principles and practical considerations described above were illustrated 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  
20            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).  
25            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

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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).

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**Digital Analysis of DNA from stool.** As a more practical example, we analyzed the DNA from stool specimens of 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*

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5 sequence (Fig. 5). 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).

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CLAIMS

1. A method for determining the ratio of a selected genetic sequence in a population of genetic sequences, comprising the steps of:

5 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;

10 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 biological sample.

15 2. The method of claim 1 wherein the step of diluting is performed until at least one-tenth 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 for the step of analyzing to determine the presence of the selected genetic sequence.

20 3. 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.

25 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 10 nucleic acid template molecules containing the reference genetic sequence.

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- 5            5.     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 the reference genetic sequence.
6.     The method of claim 1 wherein the biological sample is cell-free.
7.     The method of claim 1 wherein the number of assay samples within the set is greater than 10.
- 10           8.     The method of claim 1 wherein the number of assay samples within the set is greater than 50.
9.     The method of claim 1 wherein the number of assay samples within the set is greater than 100.
- 15           10.    The method of claim 1 wherein the number of assay samples within the set is greater than 500.
11.    The method of claim 1 wherein the number of assay samples within the set is greater than 1000.
12.    The method of claim 1 wherein the step of amplifying and the step of analyzing are performed on assay samples in the same receptacle.
- 20           13.    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.
- 25           14.    The method of claim 1 wherein the step of analyzing employs gel electrophoresis.
15.    The method of claim 1 wherein the step of analyzing employs hybridization to at least one nucleic acid probe.

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16. The method of claim 1 wherein the step of analyzing employs hybridization to at least two nucleic acid probe.
17. The method of claim 13 wherein two molecular beacon probes are used, each having a different photoluminescent dye.
- 5 18. The method of claim 13 wherein the molecular beacon probe detects a wild-type selected genetic sequence better than a mutant selected genetic sequence.
19. The method of claim 1 wherein the step of amplifying employs a single pair of primers.
- 10 20. The method of claim 1 wherein the step of amplifying employs a polymerase which is activated only after heating.
21. The method of claim 1 wherein the step of amplifying employs at least 40 cycles of heating and cooling.
22. The method of claim 1 wherein the step of amplifying employs at least 50 cycles of heating and cooling.
- 15 23. The method of claim 1 wherein the step of amplifying employs at least 60 cycles of heating and cooling.
24. The method of claim 1 wherein the biological sample is selected from the group consisting of stool, blood, and lymph nodes.
- 20 25. 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.
26. The method of claim 1 wherein the selected genetic sequence is a translocated allele.
- 25 27. The method of claim 1 wherein the selected genetic sequence is a wild-type allele.

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28. The method of claim 1 wherein the selected genetic sequence is within an amplicon which is amplified during neoplastic development.

29. The method of claim 1 wherein the selected genetic sequence is a rare exon sequence.

5 30. The method of claim 1 wherein the nucleic acid template molecules comprise cDNA of RNA transcripts and the selected genetic sequence is present on a cDNA of a first transcript and the reference genetic sequence is present on a cDNA of a second transcript.

10 31. The method of claim 1 wherein the selected genetic sequence comprises a first mutation and the reference genetic sequence comprises a second mutation.

32. The method of claim 1 wherein the selected genetic sequence and the reference genetic sequence are on distinct chromosomes.

15 33. A molecular beacon probe comprising:  
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, wherein the loop consists of 16 base pairs, wherein the loop has a  $T_m$  of 50-51°C and the stem consists of 4 base pairs having a sequence 5'-CACG-3'.

20 34. The probe of claim 33 wherein the molecular beacon probe detects a wild-type selected genetic sequence better than a mutant selected genetic sequence.

35. The probe of claim 33 wherein the molecular beacon probe detects a mutant genetic sequence better than a wild-type genetic sequence.

25 36. A molecular beacon probe comprising:  
an oligonucleotide with a stem-loop structure having a photoluminescent dye at one of the 5' or 3' ends and a quenching agent at

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the opposite 5' or 3' end, wherein the loop consists of 19-20 base pairs, wherein the loop has a  $T_m$  of 54-56°C and the stem consists of 4 base pairs having a sequence 5'-CACG-3'.

~~37.~~ A pair of molecular beacon probes comprising:

5 a first molecular beacon probe which is an oligonucleotide with a stem-loop structure having a first photoluminescent dye at one of the 5' or 3' ends and a quenching agent at the opposite 5' or 3' end, wherein the loop consists of 16 base pairs having a  $T_m$  of 50-51°C and the stem consists of 4 base pairs having a sequence 5'-CACG-3'; and

10 a second molecular beacon probe which is an oligonucleotide with a stem-loop structure having a second photoluminescent dye at one of the 5' or 3' ends and a quenching agent at the opposite 5' or 3' end, wherein the loop consists of 19-20 base pairs having a  $T_m$  of 54-56°C and the stem consists of 4 base pairs having a sequence 5'-CACG-3';

15 wherein the first and the second photoluminescent dyes are distinct.

~~38.~~ A method for determining the ratio of a selected genetic sequence in a population of genetic sequences, comprising the steps of:

20 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;

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;

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50. The method of claim 45 wherein the molecular beacon probe detects a wild-type selected genetic sequence better than a mutant selected genetic sequence.

51. The method of claim 38 wherein the step of amplifying employs a single pair of primers.

52. The method of claim 38 wherein the step of amplifying employs a polymerase which is activated only after heating.

53. The method of claim 38 wherein the step of amplifying employs at least 40 cycles of heating and cooling.

54. The method of claim 38 wherein the step of amplifying employs at least 50 cycles of heating and cooling.

55. The method of claim 38 wherein the step of amplifying employs at least 60 cycles of heating and cooling.

56. The method of claim 38 wherein the template molecules are obtained from a body sample selected from the group consisting of stool, blood, and lymph nodes.

57. The method of claim 38 wherein the template molecules are obtained from a body sample of a leukemia or lymphoma patient who has received anti-cancer therapy, said body sample being selected from the group consisting of blood and bone marrow.

58. The method of claim 38 wherein the selected genetic sequence is a translocated allele.

59. The method of claim 38 wherein the selected genetic sequence is a wild-type allele.

60. The method of claim 38 wherein the selected genetic sequence is within an amplicon which is amplified during neoplastic development.

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61. The method of claim 38 wherein the selected genetic sequence is a rare exon sequence.

5 62. The method of claim 38 wherein the nucleic acid template molecules comprise cDNA of RNA transcripts and the selected genetic sequence is present on a cDNA of a first transcript and the reference genetic sequence is present on a cDNA of a second transcript.

63. The method of claim 38 wherein the selected genetic sequence comprises a first mutation and the reference genetic sequence comprises a second mutation.

10 64. The method of claim 38 wherein the selected genetic sequence and the reference genetic sequence are on distinct chromosomes.

*add a* 

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*3/6*

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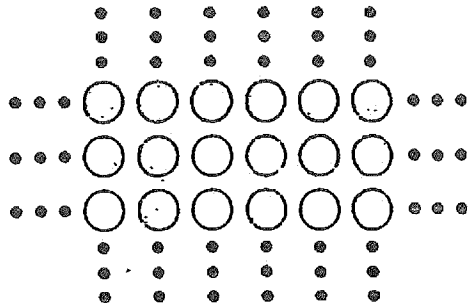
Fig. 1A

DNA

Step 1



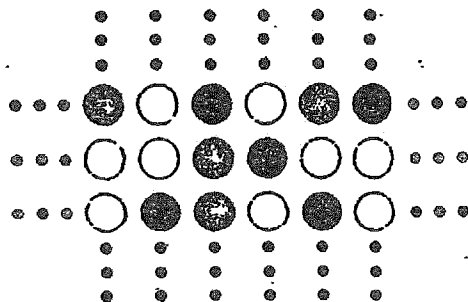
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PCR



Step 2



Add Fluorescent Probes  
Fluorometry

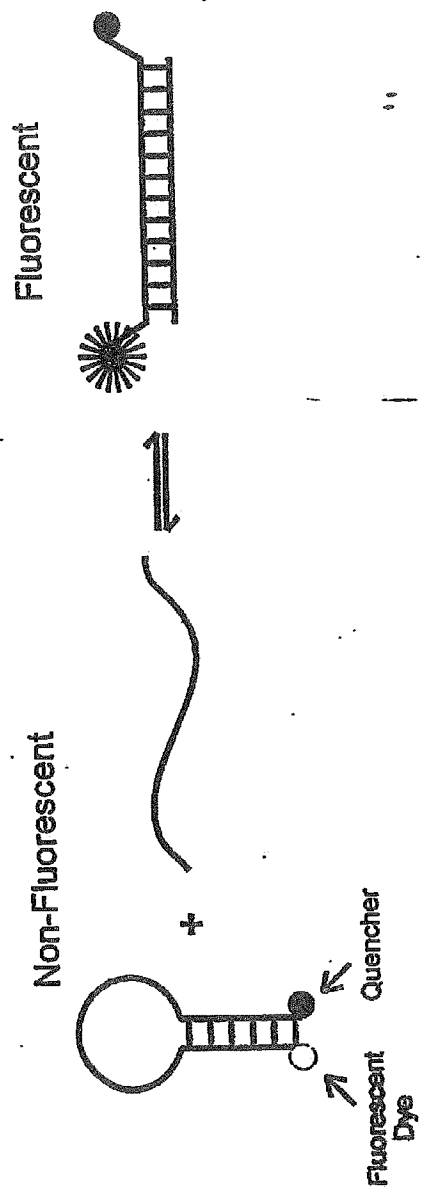


- = No PCR Product
- = Wild Type PCR Product
- ◐ = Mutant PCR Product

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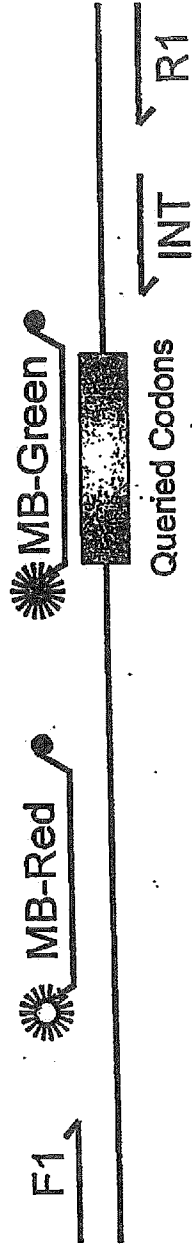
Fig. 1B



001140-92821960

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AS ORIGINALLY FILED

Fig. 1C



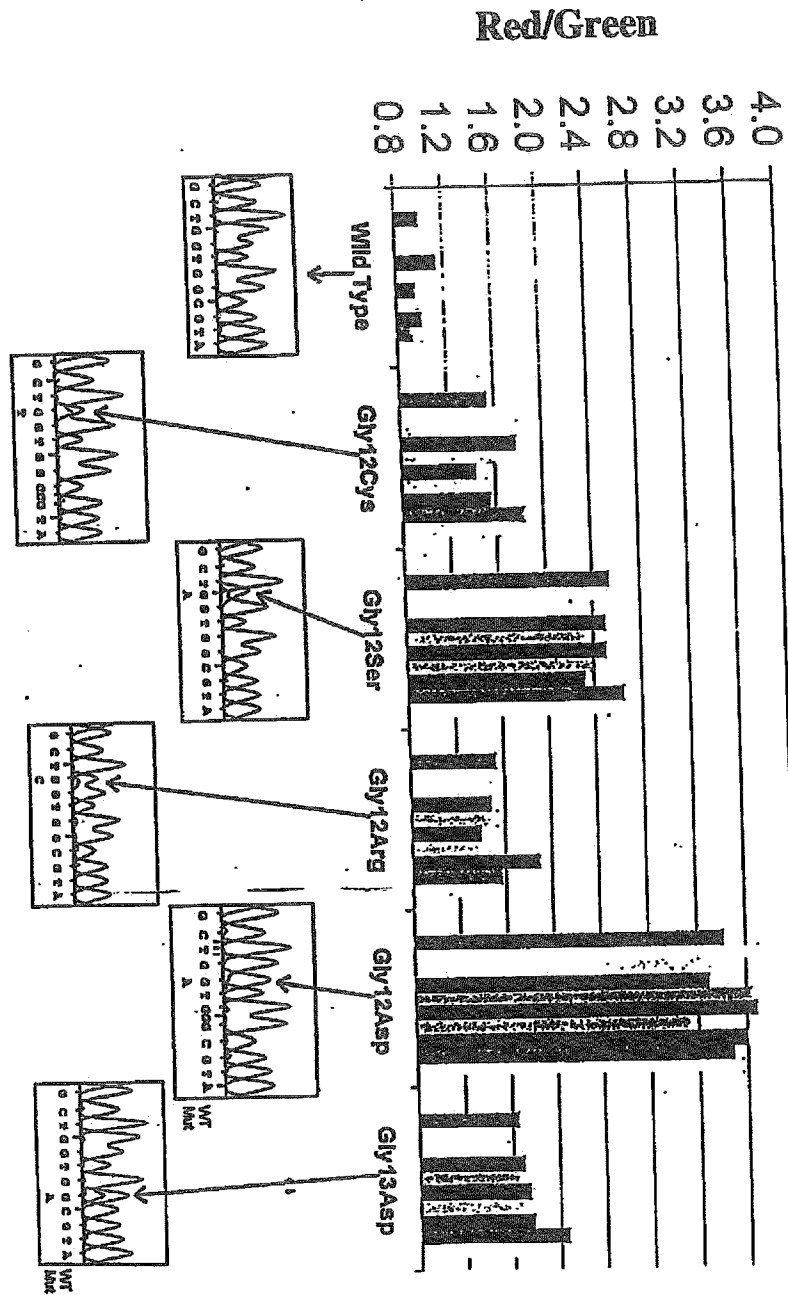
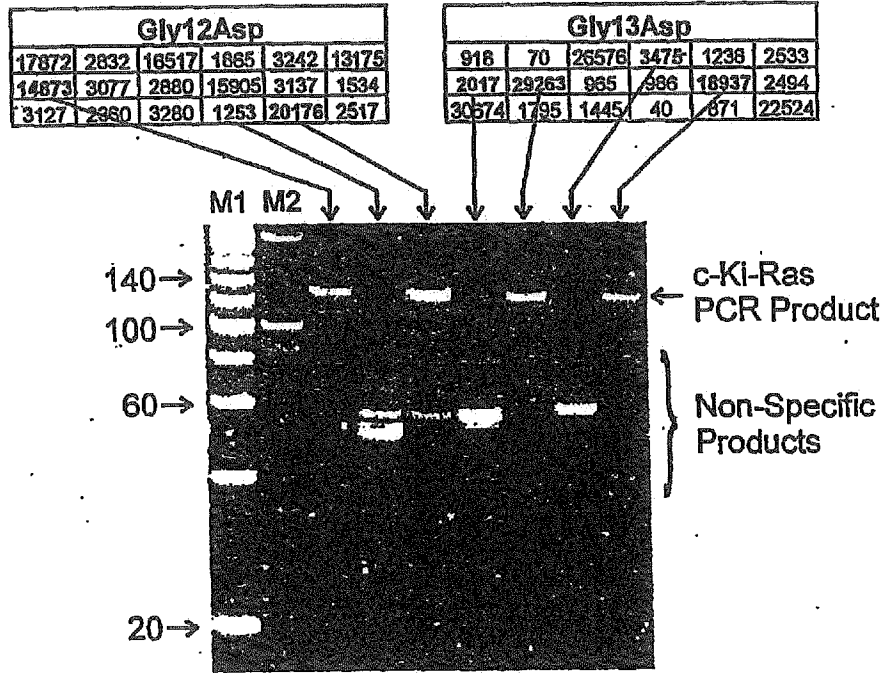


Fig. 2

09613826-071100

Fig. 3



09813025-071100

Fig. 4

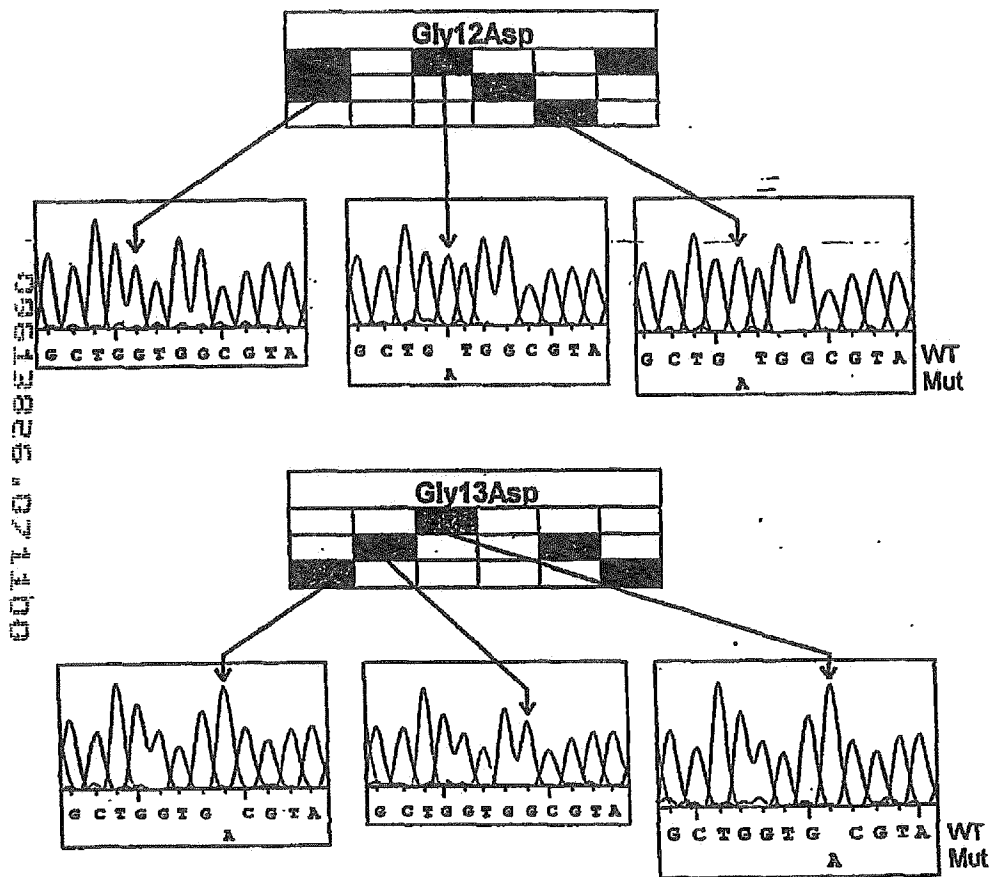
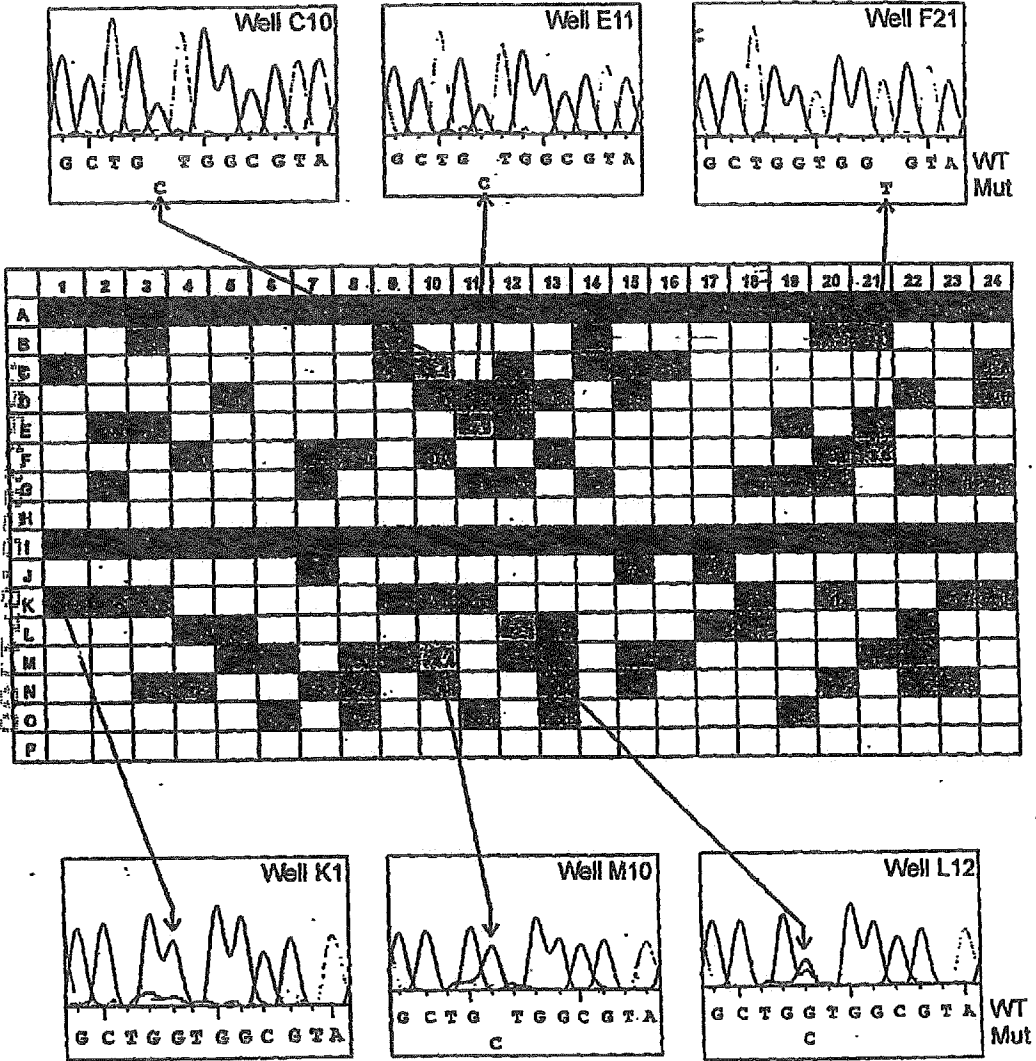


Fig. 5





07/11/00  
130003 U.S. PTO  
Page 1

NEW UNITED STATES UTILITY PATENT APPLICATION  
under 37 C.F.R. 1.53(b)

130003 U.S. PTO  
09/16/00  
07/11/00

Atty. Docket No. 01107.00031

Assistant Commissioner of Patents  
Box Patent Applications  
Washington, D.C. 20231

Enclosed herewith is a new patent application and the following papers:

First Named Inventor (or application identifier): Kenneth W. Kinzler

Title of Invention: DIGITAL AMPLIFICATION

1.  Specification 32 pages (including specification, claims, abstract) / 64 claims (5 independent)
2.  Declaration/Power of Attorney is:  
 attached in the regular manner.  
 NOT included, but deferred under 37 C.F.R. § 1.53(f).
3.  7 Distinct sheets of  Formal  Informal Drawings
4.  Preliminary Amendment.
5.  Information Disclosure Statement  
 Form 1449  
 A copy of each cited prior art reference
6.  Assignment with Cover Sheet.
7.  Priority is hereby claimed under 35 U.S.C. § 119 based upon the following application(s):

09613825-07400

| Country | Application Number | Date of Filing (day, month, year) |
|---------|--------------------|-----------------------------------|
| US      | 60/146,792         | August 2, 1999                    |

8.  Priority document(s).
9.  Statement Claiming Small Entity Status.
10.  Microfiche Computer Program (Appendix).
11.  Nucleotide and/or Amino Acid Sequence Submission.  
 Computer Readable Copy.  
 Paper Copy (identical to computer copy).  
 Statement verifying identity of above copies.

**NEW UNITED STATES UTILITY PATENT APPLICATION**  
under 37 C.F.R. 1.53(b)

Page 2

Atty. Docket No. 01107.00031

12. Calculation of Fees:

| FEES FOR                                                         | EXCESS CLAIMS        | FEE    | AMOUNT DUE |
|------------------------------------------------------------------|----------------------|--------|------------|
| Basic Filing Fee (37 C.F.R. § 1.16(a))                           |                      |        | \$690.00   |
| Total Claims in Excess of 20 (37 C.F.R. § 1.16(c))               | 44                   | 18.00  | \$792.00   |
| Independent Claims in Excess of 3 (37 C.F.R. § 1.16(b))          | 2                    | 78.00  | \$156.00   |
| Multiple Dependent Claims (37 C.F.R. § 1.16(d))                  | 0                    | 260.00 | \$0.00     |
| Subtotal - Filing Fee Due                                        |                      |        | \$1,638.00 |
|                                                                  | REDUCE BY (%)   (\$) |        |            |
| Reduction by 50%, if Small Entity (37 C.F.R. §§ 1.9, 1.27, 1.28) | 0                    |        | \$819.00   |
| <b>TOTAL FILING FEE DUE</b>                                      |                      |        | \$819.00   |
| Assignment Recordation Fee (if applicable) (37 C.F.R. § 1.21(h)) | 0                    | 40.00  | \$0.00     |
| <b>GRAND TOTAL DUE</b>                                           |                      |        | \$819.00   |

09613826-071400

13. PAYMENT is:

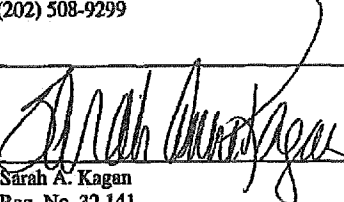
- included in the amount of the GRAND TOTAL by our enclosed check. A general authorization under 37 C.F.R. § 1.25(b), second sentence, is hereby given to credit or debit our Deposit Account No. 19-0733 for the instant filing and for any other fees during the pendency of this application under 37 C.F.R. §§ 1.16, 1.17 and 1.18.
- not included, but deferred under 37 C.F.R. § 1.53(f).

14. All correspondence for the attached application should be directed to:

Banner & Witcoff, Ltd.  
1001 G Street, N.W.  
Washington, D. C. 20001-4597  
Telephone: (202) 508-9100  
Facsimile: (202) 508-9299

15. Other: \_\_\_\_\_

Date: July 11, 2000

By:   
Sarah A. Kagan  
Reg. No. 32,141

SAK/ama



## UNITED STATES PATENT AND TRADEMARK OFFICE

 COMMISSIONER FOR PATENTS  
 UNITED STATES PATENT AND TRADEMARK OFFICE  
 WASHINGTON, D.C. 20231  
 www.uspto.gov

| APPLICATION NUMBER | FILING/RECEIPT DATE | FIRST NAMED APPLICANT | ATTORNEY DOCKET NUMBER |
|--------------------|---------------------|-----------------------|------------------------|
| 09/613,826         | 07/11/2000          | Kenneth W. Kinzler    | 01107.00031            |

 Banner & Witcoff Ltd  
 1001 G Street N W  
 Washington, DC 20001-4597

## FORMALITIES LETTER



\*0C00000005521419\*

Date Mailed: 11/01/2000

## NOTICE TO FILE MISSING PARTS OF NONPROVISIONAL APPLICATION

FILED UNDER 37 CFR 1.63(b)

## Filing Date Granted

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 and pay any fees required 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).

- The statutory basic filing fee is missing.  
Applicant must submit \$ 630 to complete the basic filing fee and/or file a small entity statement claiming such status (37 CFR 1.27).
- Total additional claim fee(s) for this application is \$948.
  - \$792 for 44 total claims over 20.
  - \$156 for 2 independent claims over 3 .
- The oath or declaration is missing.  
A properly signed oath or declaration in compliance with 37 CFR 1.63, identifying the application by the above Application Number and Filing Date, is required.
- To avoid abandonment, a late filing fee or oath or declaration surcharge as set forth in 37 CFR 1.18(e) of \$130 for a non-small entity, must be submitted with the missing items identified in this letter.
- The balance due by applicant is \$ 1768.

A copy of this notice **MUST** be returned with the reply.

*Ullman*  
 Customer Service Center  
 Initial Patent Examination Division (703) 308-1202

PART 3 - OFFICE COPY



SECTOR  
PATENT  
#3

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

|                        |   |                        |
|------------------------|---|------------------------|
| In re Application of   | ) | Group Art Unit:        |
| Bert Vogelstein et al. | ) | Examiner:              |
| Serial No. 09/613,826  | ) | Docket No. 01107.00031 |
| Filed: July 11, 2000   | ) |                        |



FOR: DIGITAL AMPLIFICATION

SUBMISSION OF EXECUTED DECLARATION FOR PATENT APPLICATION AND FILING FEES

Assistant Commissioner for Patents  
Washington, D.C. 20231

Sir:

Attached is an executed Declaration for Patent Application in compliance with the Notice to File Missing Parts of Application (copy enclosed), mailed November 1, 2000. Accordingly, it is respectfully submitted that this application is entitled to a filing date of July 11, 2000, the date upon which the specification and drawings were received by the U.S. Patent and Trademark Office. Applicants claims small entity status.

Please charge \$896.00 for filing fees to our Deposit Account No. 19-0733. The calculation is as follows:

|                                                         |                 |
|---------------------------------------------------------|-----------------|
| Basic Fee (total claims = 64)                           | \$355.00        |
| Total Claims in Excess (44)                             | 396.00          |
| Independent Claims over Three (2)                       | 80.00           |
| Surcharge for subsequent filing of executed Declaration | 65.00           |
| <b>TOTAL FILING FEE</b>                                 | <b>\$896.00</b> |

In the event any variance exists between the amount enclosed and the Patent Office charges, please charge or credit any difference to our Deposit Account No. 19-0733.

Respectfully submitted,

Date: December 12, 2000

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

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

(202) 508-9100  
SAK/ama

**DECLARATION FOR PATENT APPLICATION**



As the inventor, we hereby declare that:

Our residence and 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<br>(day month year) | Date of Issue<br>(day month year) | Priority Claimed<br>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<br>(day month year) | Priority Claimed<br>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<br>(Day, Month, Year) | Status — Patented,<br>Pending, Abandoned |
|------------------------|--------------------------------------|------------------------------------------|
|                        |                                      |                                          |







UNITED STATES PATENT AND TRADEMARK OFFICE

COMMISSIONER FOR PATENTS  
UNITED STATES PATENT AND TRADEMARK OFFICE  
WASHINGTON, D.C. 20231  
www.uspto.gov

| APPLICATION NUMBER | FILING/RECEIPT DATE | FIRST NAMED APPLICANT | ATTORNEY DOCKET NUMBER |
|--------------------|---------------------|-----------------------|------------------------|
| 09/613,826         | 07/11/2000          | Kenneth W. Kinzler    | 01107.00031            |

Banner & Witcoff Ltd  
1001 G Street N W  
Washington, DC 20001-4597



FORMALITIES LETTER



Date Mailed: 11/01/2000

NOTICE TO FILE MISSING PARTS OF NONPROVISIONAL APPLICATION

FILED UNDER 37 CFR 1.63(b)

Filing Date Granted

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 and pay any fees required 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).

- The statutory basic filing fee is missing.  
*Applicant must submit \$ 690 to complete the basic filing fee and/or file a small entity statement claiming such status (37 CFR 1.27).*
- Total additional claim fee(s) for this application is \$948.
  - \$792 for 44 total claims over 20.
  - \$166 for 2 independent claims over 3 .
- The oath or declaration is missing.  
*A properly signed oath or declaration in compliance with 37 CFR 1.63, identifying the application by the above Application Number and Filing Date, is required.*
- To avoid abandonment, a late filing fee or oath or declaration surcharge as set forth in 37 CFR 1.16(e) of \$130 for a non-small entity, must be submitted with the missing items identified in this letter.
- The balance due by applicant is \$ 1768.

A copy of this notice **MUST** be returned with the reply

*Handwritten signature*

Customer Service Center  
Initial Patent Examination Division (703) 308-1202

PART 2 - COPY TO BE RETURNED WITH RESPONSE

09613826  
 06000097 150733  
 155.00 CH  
 160.00 CH  
 398.00 CH  
 12/19/2000  
 01 01-201  
 02 02-202  
 03 03-203  
 04 04-204

#15

PATENT

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re Application of  
Bert Vogelstein et al.  
Serial No. 09/613,826  
Filed: July 11, 2000



)  
) Attn: Application Branch  
)  
) Atty. Dkt. No. 01107.00031

For: **DIGITAL AMPLIFICATION**

INFORMATION DISCLOSURE STATEMENT

The Honorable Commissioner  
of Patents and Trademarks  
Washington, D.C. 20231

Sir:

In accordance with 37 C.F.R. §§ 1.97 and 1.98, enclosed is a PTO Form-1449 listing documents for consideration by the Examiner during the prosecution of the subject application and a copy of each of the identified documents. It is believed no fee is required to make this a complete and timely filing. However, if a fee is required, please charge our Deposit Account No. 19-0733.

Consideration of this information is respectfully requested.

Respectfully submitted,

Date: December 12, 2000

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

Banner & Witcoff, Ltd.  
1001 G Street, N.W., Eleventh Floor  
Washington, D.C. 20001-4597  
(202) 508-9100  
SAK/ama

## File History Report

Paper number \_\_\_\_\_ is missing from the United States Patent Trademark Office's copy of the file History. No additional information is available.

The following page(s) 1 of 2 PTO -1449 of paper number 4 is/are missing from the United States Patent and Trademark Office's original copy of the file history. No additional information is available

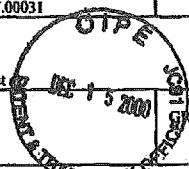
The following checked item(s) below of paper number \_\_\_\_\_ is/are missing from the United States Patent and Trademark Office's original copy of the file history. No additional information is available

- PTO 1449
- PTO 892
- PTO 948
- PTO 1474
- Assignment
- Cover page

Additional comments: \_\_\_\_\_

#4

Sheet 2 of 2

|                                                                                                                                       |                                                                                    |                             |
|---------------------------------------------------------------------------------------------------------------------------------------|------------------------------------------------------------------------------------|-----------------------------|
| PTO-1449 (Modified)<br>U.S. DEPARTMENT OF COMMERCE<br>PATENT AND TRADEMARK OFFICE<br>INFORMATION DISCLOSURE STATEMENT<br>BY APPLICANT | ATTY. DOCKET NO.<br>01107.00031                                                    | SERIAL NUMBER<br>09/613,826 |
|                                                                                                                                       | APPLICANT<br>Bert Vogelstein et al                                                 |                             |
|                                                                                                                                       | FILING DATE<br>July 11, 2000                                                       | GROUP ART UNIT<br>1637      |
|                                                                                                                                       |  |                             |





U.S. PATENT DOCUMENTS


| EXAMINER INITIAL | DOCUMENT NUMBER | DATE | NAME | CLASS | SUB CLASS | FILING DATE |
|------------------|-----------------|------|------|-------|-----------|-------------|
|                  |                 |      |      |       |           |             |
|                  |                 |      |      |       |           |             |
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|                  |                 |      |      |       |           |             |

FOREIGN PATENT DOCUMENTS

| EXAMINER INITIAL | DOCUMENT NUMBER | DATE | COUNTRY | CLASS | SUB CLASS | TRANSLATION YEAR |
|------------------|-----------------|------|---------|-------|-----------|------------------|
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|                  |                 |      |         |       |           |                  |
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|                  |                 |      |         |       |           |                  |
|                  |                 |      |         |       |           |                  |
|                  |                 |      |         |       |           |                  |

OTHER DOCUMENTS (Including Author, Title, Date, Pertinent Pages, Etc.)

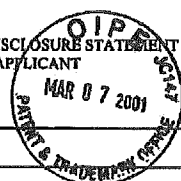
|                                                                                     |                                                                                                                                                                                                                                                                                      |
|-------------------------------------------------------------------------------------|--------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------|
|  | Eickard et al. "Mutation detection and single-molecule counting using isothermal rolling-circle amplification" <i>Nature Genetics</i> , Vol. 10, pages 325-32                                                                                                                        |
|  | 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" <i>Forensic Science International</i> 66 (1994) pages 129-141 |
|  | Li "Amplification and analysis of DNA sequences in single human sperm and diploid cells" <i>Nature</i> Vol. 335 September 29, 1988 pages 414-417                                                                                                                                     |
|  | Zhang "Whole genome amplification from a single cell: Implications for genetic analysis" <i>Proc. Natl. Acad. Sc. USA</i> , Vol 89 pages 5847-5851 July 1992                                                                                                                         |
|                                                                                     |                                                                                                                                                                                                                                                                                      |
|                                                                                     |                                                                                                                                                                                                                                                                                      |

|                                                                                                                                                                                                          |                           |
|----------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------|---------------------------|
| EXAMINER<br>                                                                                                          | DATE CONSIDERED<br>4/5/01 |
| EXAMINER: Initial citation if reference was considered. Draw line through citation if not in conformance to MFEP 609 and not considered. Include copy of this form with next communication to applicant. |                           |



#5

|                                                                                                                                       |                                       |                                                 |
|---------------------------------------------------------------------------------------------------------------------------------------|---------------------------------------|-------------------------------------------------|
| PTO-1449 (Modified)<br>U.S. DEPARTMENT OF COMMERCE<br>PATENT AND TRADEMARK OFFICE<br>INFORMATION DISCLOSURE STATEMENT<br>BY APPLICANT | ATTY. DOCKET NO.<br>01107.00031       | SERIAL NUMBER <i>09613926</i><br><del>78A</del> |
|                                                                                                                                       | APPLICANTS<br>Bert Vogelstein, et al. |                                                 |
|                                                                                                                                       | FILING DATE<br>July 11, 2000          | GROUP ART UNIT<br><del>78A</del> 1637           |
|                                                                                                                                       | MAR 08 2001<br>TECH CENTER 1600/2900  |                                                 |



U.S. PATENT DOCUMENTS

| EXAMINER INITIAL | DOCUMENT NUMBER | DATE    | NAME            | CLASS | SUB CLASS | FILING DATE |
|------------------|-----------------|---------|-----------------|-------|-----------|-------------|
| <i>JS</i>        | 5,670,325       | 9/1997  | Lapidus, et al. | —     | —         |             |
| <i>J</i>         | 5,928,870       | 7/1999  | Lapidus, et al. | —     | —         |             |
| <i>J</i>         | 6,020,137       | 2/2000  | Lapidus, et al. | —     | —         |             |
| <i>JS</i>        | 6,143,496       | 11/2000 | Brown, et al.   | —     | —         |             |

OTHER DOCUMENTS (Including Author, Title, Date, Pertinent Pages, Etc.)

|           |                                                                                                                                                                                                                                                                                                 |
|-----------|-------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------|
| <i>JS</i> | Darren G. MONCKTON, et al., "Minisatellite "Isocalle" Discrimination in Pseudohomozygotes by Single Molecule PCR and Variant Repeat Mapping", <i>Genomics</i> 11, pp. 465-467, 1991                                                                                                             |
| <i>JS</i> | Gualberto RUANO, et al., "Haplotype of Multiple Polymorphisms Resolved by Enzymatic Amplification of Single DNA Molecules", <i>Proc. National Science USA</i> , 1990 (6) - 87 pp 6276-6280                                                                                                      |
| <i>JS</i> | W. NAVIDI, et al., "Using PCR in Preimplantation Genetic Disease Diagnosis", <i>Human Reproduction</i> , Vol. 6, No. 6, pp. 836-849, 1991                                                                                                                                                       |
| <i>JS</i> | Hongus LI, et al., "Amplification and Analysis of DNA Sequences in Single Human Sperm and Diploid Cells", <i>Nature</i> , Vol. 335, September 29, 1988 pp 414-417                                                                                                                               |
| <i>JS</i> | Ramon PARSONS, et al., "Mismatch Repair Deficiency in Phenotypically Normal Human Cells", <i>Science</i> , Vol. 268, May 5, 1995 pp 738-740                                                                                                                                                     |
| <i>JS</i> | Lin ZHANG, et al., "Whole Genome Amplification from a Single Cell: Implications for Genetic Analysis", <i>Proc. National Science USA</i> , Vol. 89, pp. 5847-5851, July 1992                                                                                                                    |
| <i>JS</i> | David SIDRANSKY, et al., "Clonal Expansion of p53 Mutant Cells is Associated with Brain Tumour Progression", <i>Nature</i> , February 27, 1992 vol. 355, pp 816-817                                                                                                                             |
| <i>JS</i> | Alec J. Jeffreys, et al., "Mutation Processes at Human Minisatellites", <i>Electrophoresis</i> , pp. 1577-1585, 1995                                                                                                                                                                            |
| <i>JS</i> | 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", <i>Forensic Science International</i> , Vol. 66, pp. 129-141, 1994 |
| <i>JS</i> | Paul M. LIZARDI, et al., "Mutation Detection and Single-Molecule Counting Using Isothermal Rolling-Circle Amplification", <i>Nature Genetics</i> , Vol. 19, July 1998 pp 225-232                                                                                                                |
| <i>JS</i> | W. NAVIDI, et al., "Using PCR in Preimplantation Genetic Disease Diagnosis", <i>Human Reproduction</i> , Vol. 6, 1991                                                                                                                                                                           |
| <i>JS</i> | Hongus LI, et al., "Amplification and Analysis of DNA Sequences in Single Human Sperm and Diploid Cells" <i>Nature</i> , Vol. 335, September 29, 1988                                                                                                                                           |

|                                                                                                                                                                                                          |                               |
|----------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------|-------------------------------|
| EXAMINER <i>Jeffrey Sui</i>                                                                                                                                                                              | DATE CONSIDERED <i>4/5/01</i> |
| EXAMINER: Initial citation if reference was considered. Draw line through citation if not in conformance to MPEP 609 and not considered. Include copy of this form with next communication to applicant. |                               |



UNITED STATES DEPARTMENT OF COMMERCE  
United States Patent and Trademark Office  
Address: COMMISSIONER OF PATENTS AND TRADEMARKS  
Washington, D.C. 20231

| APPLICATION NO. | FILING DATE | FIRST NAMED INVENTOR | ATTORNEY DOCKET NO. |
|-----------------|-------------|----------------------|---------------------|
| 09/613,826      | 07/11/00    | VOGELSTEIN           | E 01107.00031       |

022907  
BANNER & WITCOFF  
1001 G STREET N W  
SUITE 1100  
WASHINGTON DC 20001

HM22/0412

EXAMINER  
SIEW, J

ART UNIT      PAPER NUMBER  
1656              6

DATE MAILED:      04/12/01

Please find below and/or attached an Office communication concerning this application or proceeding.

Commissioner of Patents and Trademarks

|                              |                               |                                   |  |
|------------------------------|-------------------------------|-----------------------------------|--|
| <b>Office Action Summary</b> | Application No.<br>09/613,826 | Applicant(s)<br>VOGELSTEIN ET AL. |  |
|                              | Examiner<br>Jeffrey Slew      | Art Unit<br>1656                  |  |

-- 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) 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 the period for reply specified above is less than thirty (30) days, a reply within the statutory minimum of thirty (30) days will be considered timely.
- 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 07 March 2001.

2a)  This action is FINAL.                      2b)  This action is non-final.

3)  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**

4)  Claim(s) 1-64 is/are pending in the application.

    4a) Of the above claim(s) \_\_\_\_\_ is/are withdrawn from consideration.

5)  Claim(s) \_\_\_\_\_ is/are allowed.

6)  Claim(s) 1-64 is/are rejected.

7)  Claim(s) \_\_\_\_\_ is/are objected to.

8)  Claims \_\_\_\_\_ are subject to restriction and/or election requirement.

**Application Papers**

9)  The specification is objected to by the Examiner.

10)  The drawing(s) filed on \_\_\_\_\_ is/are objected to by the Examiner.

11)  The proposed drawing correction filed on \_\_\_\_\_ is: a)  approved b)  disapproved.

12)  The oath or declaration is objected to by the Examiner.

**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.

14)  Acknowledgment is made of a claim for domestic priority under 35 U.S.C. § 119(e).

**Attachment(s)**

15)  Notice of References Cited (PTO-892)

16)  Notice of Draftsperson's Patent Drawing Review (PTO-948)

17)  Information Disclosure Statement(s) (PTO-1449) Paper No(s) 4 & 6.

18)  Interview Summary (PTO-413) Paper No(s) \_\_\_\_\_.

19)  Notice of Informal Patent Application (PTO-152)

20)  Other: *notice to comply*.



**DETAILED ACTION**

1. This application contains sequence disclosures that are encompassed by the definitions for nucleotide and/or amino acid sequences set forth in 37 CFR 1.821(a)(1) and (a)(2). However, this application fails to comply with the requirements of 37 CFR 1.821 through 1.825 for the reason(s) set forth on the attached Notice To Comply With Requirements For Patent Applications Containing Nucleotide Sequence And/Or Amino Acid Sequence Disclosures.

APPLICANT IS GIVEN THE RESPONSE PERIOD SET FORTH IN THIS OFFICE ACTION IN WHICH TO COMPLY WITH THE SEQUENCE RULES, 37 CFR 1.821 - 1.825. Failure to comply with these requirements will result in ABANDONMENT of the application under 37 CFR 1.821(g). Extensions of time may be obtained by filing a petition accompanied by the extension fee under the provisions of 37 CFR 1.136. In no case may an applicant extend the period for response beyond the six month statutory period. Applicant is requested to return a copy of the attached Notice to Comply with the response. The application is not in compliance for the reason(s) set forth on the attached Notice to Comply With the Sequence Rules or CRF Diskette Problem Report.

*Information Disclosure Statement*

2. The listing of references in the specification is not a proper information disclosure statement. 37 CFR 1.98(b) requires a list of all patents, publications, or other information submitted for consideration by the Office, and MPEP § 609 A(1) states, "the list may not be incorporated into the specification but must be submitted in a separate paper." Therefore, unless

Application/Control Number: 09/613,826  
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the references have been cited by the examiner on form PTO-892, they have not been considered.

*Specification*

3. In the Brief Description of the Drawings Figure 1 is referred to but no Figure 1 exists in the Drawings. The specification should be amended to recite the actual figures in the drawings i.e. Figure 1A, 1B and 1C.

4. Moreover, the specification contains nucleotide sequences which require Sequence identifiers (see page 14 line 30 & 31). Appropriate correction is required (see also Notice to comply).

*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.

Claims 1-64 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.

A) Claims 1-32 & 38-64 lack rejected under 35 U.S.C. 112, second paragraph, as being incomplete for omitting essential steps, such omission amounting to a gap between the steps. See MPEP § 2172.01. The omitted steps are: serially diluting to form a set of assay samples and testing by PCR. The specification provides guidance as to determining the analyte concentration in which the samples are serially diluted and the concentration is determined by PCR (see page 13 line 19). It appears that the initial concentration of sample at the start of the assay is essential to the invention. Such a step would be critical because it is unclear as to how otherwise the initial concentration would be achieved without testing by PCR.

B) Claims 1-32 & 38-64 lack rejected under 35 U.S.C. 112, second paragraph, as being incomplete for omitting essential steps, such omission amounting to a gap between the steps. See MPEP § 2172.01. The omitted steps are: linear amplification by PCR. The step of linear amplification appears essential to the invention (see page 14 line 18).

C) The use of the term "consists" is confusing in claims 33, 36 & 37 rendering claims 33-37 indefinite. It cannot be determined whether the claim intends open or closed language for the limitation of the sequence. Proper Markush language is required.

D) Claim 2 is confusing because it is unclear as to whether each sample of the fraction of one out ten are to contain N molecules.

*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 negated by the manner in which the invention was made.

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).

5. Claims 1,3,4-11,14-16 & 19-32 are rejected under 35 U.S.C. 103(a) as being unpatentable over Lapidus et al (US5,928,870 July 27, 1999) in view of Ruano et al (PNAS vol. 87 pp. 6296-63000 August 1990).

Lapidus et al teach a method of determining the subpopulation of genomically transformed cells such as in stool samples by enumerating number molecules of a target sequence and comparing with a number of molecules of a reference genomic sequence (see whole doc. esp. col.2 lines 58-66). They teach statistical difference leads to differences in genomic sequence (see col. 2 lines 8-10). They teach that the reference and target are different genetic loci (see col. 7 lines 63-65). They perform amplification by PCR and detect by probing (see col. 11 lines 18-51 & 40-45). They teach that one probe is to wild type genome (see col. 5 lines 40-46). They test malignant cells and the method would be useful for precancerous cells in humans and colorectal cancer (see col. 5 line 30-35). They teach that method would be useful for studying patients (see col. 6 line 17-20).

Lapidus et al do not teach dilution to one half genomic equivalent in samples.

Ruano et al teach single molecule dilution (SMD) in which genomic DNA concentration is one haploid equivalent per aliquot (see whole doc. esp. pp. 6296 & Fig. 3).

One of ordinary skill would have been motivated to apply Ruano et al SMD method to Lapidus et al's comparison method in order to determine actual allele concentration ratios. Ruano et al state that SMD method avoids the empirical optimization of amplification conditions and allows resolution of ambiguous arrangement of polymorphic markers by isolating into definitive haplotypes. It would have been prima facie obvious to apply Ruano et al's dilution method to Lapidus et al's method in order to accurately determine allele ratios.

Moreover, it would have been prima facie obvious to further optimize the assay conditions as in the increasing the number of PCR cycles or increasing the dilution schema to achieve single molecule dilution in order to effectively amplify from haploid equivalent.

6. Claims 12,13,17 & 18 are rejected under 35 U.S.C. 103(a) as being unpatentable over Lapidus et al (US5,928,870 July 27, 1999) in view of Ruano et al (PNAS vol. 87 pp. 6296-63000 August 1990) in further view of Tyagi et al (US5,925,517 July 20, 1999).

The teachings and suggestions of Lapidus and Ruano et al are described previously.

Lapidus et al do not teach molecular beacons.

Tyagi et al teach molecular beacons (see whole doc. & Fig. 3). They teach that the probe allows monitoring of progress of reactions that produce nucleic acids with either linear or exponential kinetics. They provide sensitive detection (see col. 4 lines 22-40).

One of ordinary skill would have been motivated to apply Tyagi et al's molecular beacons to the combined invention of Lapidus and Ruano et al's enumeration method in order to accurately monitor detection over real time. It would have been prima facie obvious to apply Tyagi et al's probes which would allow detection of the different sequences in Lapidus and Ruano et al's method in order to achieve accurate quantification.

#### SUMMARY

Claims 33-37 are free of the prior art but rejected under 112 second paragraph. There is no prior art that teach or suggest a molecular beacon probe that has a loop consisting of 16 base pairs and having a Tm of 50-51C and the stem consisting of CACG sequence.

The closest prior art is Tyagi et al (US5,925,517) who teach a molecular beacon which has 15 base pair loop but a  $T_m$  of approximately 40C ( $T_m = [(A+T) \times 2C + (G+C) \times 4C]$  (see PCR essential Data page 53 1995) and the stem is GCGAG (see col. . Tyagi et al (US6,037,130) teach molecular beacon with a stem comprising CACG (see col. 11 probe 3) but with a loop of  $T_m$  65C (see col. 28 line 54). Moreover, the prior art has been focused on the  $T_m$  of the stem which relates to the functioning of the opening and closing of the hairpin during hybridization.

Claims 2 & 38-64 is free of the prior art but rejected under 112 second paragraph. Applicant is directed to 112 second paragraph rejections concerning these claims as the lack clarity of the claims may prove a barrier to allowability. There is no prior art that teach that one tenth or one fiftieth of samples in a set comprise N molecules such that  $1/N$  is larger than the ratio of selected genetic sequence to total genetic sequences required for the step of analyzing to determine presence of selected genetic sequence.

#### CONCLUSION

Any inquiry concerning this communication or earlier communications from the examiner should be directed to Jeffrey Siew whose telephone number is (703) 305-3886 and whose e-mail address is Jeffrey.Siew@uspto.gov. However, the office cannot guarantee security through the e-mail system nor should official papers be transmitted through this route. The examiner can best be reached on Monday through Thursday from 6:30 a.m. to 4 p.m. If attempts to reach the examiner by telephone are unsuccessful, the examiner's supervisor, Gary Jones, can be reached on (703)-308-1152.

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Art Unit: 1656

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Any inquiry of a general nature or relating to the status of this application or proceeding should be directed to the receptionist for Technology Center 1600 whose telephone number is (703) 308-0196.

Papers related to this application may be submitted to Group 1600 by facsimile transmission. Papers should be faxed to Group 1600 via the PTO Fax Center located in Crystal Mall 1. The faxing of such papers must conform with the notice published in the Official Gazette, 1096 OG 30 (November 15, 1989). The CM1 Center numbers for Group 1600 are Voice (703) 308-3290 and Fax (703) 308-4556 or (703) 308-4242.

  
Jeffrey Siew

April 7, 2001



|                                   |                                       |                                                                 |             |
|-----------------------------------|---------------------------------------|-----------------------------------------------------------------|-------------|
| <b>Notice of References Cited</b> | Application/Control No.<br>09/613,828 | Applicant(s)/Patent Under<br>Reexamination<br>VOGELSTEIN ET AL. |             |
|                                   | Examiner<br>Jeffrey Siew              | Art Unit<br>2858/637                                            | Page 1 of 1 |

**U.S. PATENT DOCUMENTS**

| * |   | Document Number<br>Country Code-Number-Kind Code | Date<br>MM-YYYY | Name        | Classification |   |
|---|---|--------------------------------------------------|-----------------|-------------|----------------|---|
|   |   |                                                  |                 |             |                |   |
|   | A | US-5,826,517 -A                                  | 07-1999         | Tyagi et al | 435            | 6 |
|   | B | US-6,037,130-A                                   | 03-2000         | Tyagi et al | 435            | 6 |
|   | C | US- -                                            |                 |             |                |   |
|   | D | US- -                                            |                 |             |                |   |
|   | E | US- -                                            |                 |             |                |   |
|   | F | US- -                                            |                 |             |                |   |
|   | G | US- -                                            |                 |             |                |   |
|   | H | US- -                                            |                 |             |                |   |
|   | I | US- -                                            |                 |             |                |   |
|   | J | US- -                                            |                 |             |                |   |
|   | K | US- -                                            |                 |             |                |   |
|   | L | US- -                                            |                 |             |                |   |
|   | M | US- -                                            |                 |             |                |   |

**FOREIGN PATENT DOCUMENTS**

| * |   | Document Number<br>Country Code-Number-Kind Code | Date<br>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 | Newton Essential PCR pp.51-52 1995                                                         |
|   | V |                                                                                            |
|   | W |                                                                                            |
|   | X |                                                                                            |

\*A copy of this reference is not being furnished with this Office action. (See MPEP § 707.05(a).)  
Dates in MM-YYYY format are publication dates. Classifications may be US or foreign.



PATENT

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re Application of )  
 Bert Vogelstein et al. ) Attn: Application Branch  
 Serial No. 09/613,826 )  
 Filed: July 11, 2000 ) Atty. Dkt. No. 01107.00031

#7  
S.G.J.  
7/18/01

For: **DIGITAL AMPLIFICATION**

INFORMATION DISCLOSURE STATEMENT

The Honorable Commissioner  
of Patents and Trademarks  
Washington, D.C. 20231

Sir:

In accordance with 37 C.F.R. §§ 1.97 and 1.98, enclosed is a PTO Form-1449 listing documents for consideration by the Examiner during the prosecution of the subject application and a copy of each of the identified documents. It is believed no fee is required to make this a complete and timely filing. However, if a fee is required, please charge our Deposit Account No. 19-0733.

Consideration of this information is respectfully requested.

Respectfully submitted,

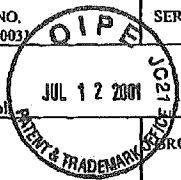
Date: December 12, 2000

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

Banner & Witcoff, Ltd.  
1001 G Street, N.W., Eleventh Floor  
Washington, D.C. 20001-4597  
(202) 508-9100  
SAK/jama

# 7

|                                                                                                                                       |                                     |                         |
|---------------------------------------------------------------------------------------------------------------------------------------|-------------------------------------|-------------------------|
| PTO-1449 (Modified)<br>U.S. DEPARTMENT OF COMMERCE<br>PATENT AND TRADEMARK OFFICE<br>INFORMATION DISCLOSURE STATEMENT<br>BY APPLICANT | ATTY. DOCKET NO.<br>01107.0003      | SERIAL NUMBER<br>613826 |
|                                                                                                                                       | APPLICANT<br>Bert Vogelstein et al. |                         |
|                                                                                                                                       | FILING DATE<br>July 11, 2000        | GROUP ART UNIT<br>1637  |



U.S. PATENT DOCUMENTS

| EXAMINER INITIAL | DOCUMENT NUMBER | DATE      | NAME           | CLASS | SUB CLASS | FILING DATE |
|------------------|-----------------|-----------|----------------|-------|-----------|-------------|
| JS               | 5,928,870       | 7/27/1999 | Lapidus et al. |       |           |             |
|                  | 5,698,523       | 9/23/1997 | Lapidus et al. |       |           |             |
| JS               | 6,020,137       | 2/4/2000  | Lapidus et al. |       |           |             |

FOREIGN PATENT DOCUMENTS

| EXAMINER INITIAL | DOCUMENT NUMBER | DATE | COUNTRY | CLASS | SUB CLASS | TRANSLATION YES/NO |
|------------------|-----------------|------|---------|-------|-----------|--------------------|
|                  |                 |      |         |       |           |                    |
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|                  |                 |      |         |       |           |                    |

OTHER DOCUMENTS (Including Author, Title, Date, Pertinent Pages, Etc.)

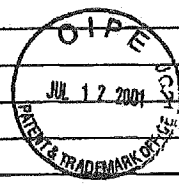
|    |                                                                                                                                                                                 |
|----|---------------------------------------------------------------------------------------------------------------------------------------------------------------------------------|
| JS | Jeffreys et al. "Mutation processes of human minisatellites" Electrophoresis 1995, 16 pages 1577-1585                                                                           |
|    | Ruano et al. "Haplotype of multiple polymorphisms resolved by enzymatic amplification of single DNA molecules" Proc. Natl. Acad. Sci. USA Vol. 87, pages 6296-6300, August 1990 |
|    | Parsons et al. "Mismatch Repair Deficiency in Phenotypically Normal Human Cells" Science, Vol. 268 May 5, 1995 pages 738-740                                                    |
|    | Macklon et al. "Minisatellite "Isoflects" Discrimination in Pseudodiploidy by Single Molecule PCR and Variant Repeat Mapping" Genomics, Vol. 11, 1991 pages 465-467             |
|    | Sidransky et al. "Clonal expansion of p53 mutant cells is associated with brain tumor progression" Nature Vol. 355, pages 846-847 1992                                          |
| JS | Navidi et al. "Using PCR in preimplantation genetic disease diagnosis" Human reproduction Vol. 6, No. 6, pages 836-849 1991                                                     |

|                                                                                                                                                                                                          |             |                 |         |
|----------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------|-------------|-----------------|---------|
| EXAMINER                                                                                                                                                                                                 | Jeffrey Liu | DATE CONSIDERED | 3/23/02 |
| EXAMINER: Initial citation if reference was considered. Draw line through citation if not in conformance to MPEP 609 and not considered. Include copy of this form with next communication to applicant. |             |                 |         |

|                                                                                                                                               |                                     |                             |
|-----------------------------------------------------------------------------------------------------------------------------------------------|-------------------------------------|-----------------------------|
| PTO-1449 (Modified)<br><br>U.S. DEPARTMENT OF COMMERCE<br>PATENT AND TRADEMARK OFFICE<br><br>INFORMATION DISCLOSURE STATEMENT<br>BY APPLICANT | ATTY. DOCKET NO.<br>01107.00031     | SERIAL NUMBER<br>09/613,826 |
|                                                                                                                                               | APPLICANT<br>Bert Vogelstein et al. |                             |
|                                                                                                                                               | FILING DATE<br>July 11, 2000        | GROUP ART UNIT<br>1637      |
|                                                                                                                                               |                                     |                             |

U.S. PATENT DOCUMENTS

| EXAMINER INITIAL | DOCUMENT NUMBER | DATE | NAME | CLASS | SUB CLASS | FILING DATE |
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FOREIGN PATENT DOCUMENTS

| EXAMINER INITIAL | DOCUMENT NUMBER | DATE | COUNTRY | CLASS | SUB CLASS | TRANSLATION YES/NO |
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|                  |                 |      |         |       |           |                    |
|                  |                 |      |         |       |           |                    |
|                  |                 |      |         |       |           |                    |

OTHER DOCUMENTS (Including Author, Title, Date, Pertinent Pages, Etc.)

|                        |                                                                                                                                                                                                                                                                               |
|------------------------|-------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------|
| [Handwritten initials] | Lizardi et al. "Mutation detection and single-molecule counting using isothermal rolling-circle amplification" Nature Genetics, Vol. 19 pages 225-32                                                                                                                          |
|                        | 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 66 (1994) pages 129-141 |
|                        | Li "Amplification and analysis of DNA sequences in single human sperm and diploid cells." Nature Vol. 335 September 29, 1988 pages 414-417                                                                                                                                    |
| [Handwritten initials] | Zhang "Whole genome amplification from a single cell: Implications for genetic analysis" Proc. Natl. Acad. Sc. USA., Vol 89 pages 5847-5851 July 1992                                                                                                                         |
|                        |                                                                                                                                                                                                                                                                               |
|                        |                                                                                                                                                                                                                                                                               |

|                                                                                                                                                                                                          |                            |
|----------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------|----------------------------|
| EXAMINER<br>[Handwritten signature: Jeffrey Bee]                                                                                                                                                         | DATE CONSIDERED<br>3/23/02 |
| EXAMINER: Initial citation if reference was considered. Draw line through citation if not in conformance to MPEP 609 and not considered. Include copy of this form with next communication to applicant. |                            |



PATENT

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

|                                   |                          |
|-----------------------------------|--------------------------|
| In re Application of              | )                        |
| Bert Vogelstein, et. al.          | ) Group Art Unit: 1656   |
| Serial No. 09/613,826             | ) Examiner: J Siew       |
| Filing Date: July 11, 2000        | ) Docket No. 01107.00031 |
| For: <b>DIGITAL AMPLIFICATION</b> |                          |

8/a  
AJS  
7/18/01

AMENDMENT

RECEIVED

JUL 17 2001

TECH CENTER 1600/2900

Assistant Commissioner for Patents  
Washington, D.C. 20231

Sir:

In response to the Office Action mailed April 12, 2001, applicants request entry of the following amendments and request reconsideration of the claims. Claims 1-64 are pending in the application. Claims 2 and 38-64 are allowable over the prior art. If any additional fee is due please change our Deposit Account No. 19-0733.

IN THE CLAIMS

Please add new claims 65-69.

65. (New) A molecular beacon probe comprising:  
 an oligonucleotide comprising a stem and a loop structure and having a

1

photoluminescent dye at one of the 5' or 3' ends and a quenching agent at the opposite 5' or 3' end, wherein the loop comprises 16 base pairs and has a T<sub>m</sub> of 50-51°C, and wherein the stem comprises 4 base pairs having a sequence 5'-CACG-3'.

66. (New) The molecular beacon probe of claim 65, wherein the probe detects a wild-type nucleic acid better than a mutant nucleic acid.

67. (New) The molecular beacon probe of claim 65, wherein the probe detects a mutant nucleic acid better than a wild-type nucleic acid.

68. (New) A molecular beacon probe comprising:  
an oligonucleotide comprising a stem and a loop structure and having a photoluminescent dye at one of the 5' or 3' ends and a quenching agent at the opposite 5' or 3' end, wherein the loop comprises 19-20 base pairs and has a T<sub>m</sub> of 54-56°C, and wherein the stem comprises 4 base pairs having a sequence 5'-CACG-3'.

al  
cont

69. (New) A pair of molecular beacon probes comprising:  
a first oligonucleotide comprising a first stem and a first loop structure and having a photoluminescent dye at one of the 5' or 3' ends and a quenching agent at the opposite 5' or 3' end, wherein the first loop comprises 16 base pairs and has a T<sub>m</sub> of 50-51°C, and wherein the first stem comprises 4 base pairs having a sequence 5'-CACG-3'; and  
a second oligonucleotide comprising a second stem and a second loop structure

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a1  
Cont

and having a photofluorescent dye at one of the 5' or 3' ends and a quenching agent at the opposite 5' or 3' end, wherein the second loop comprises 19-20 base pairs and has a Tm of 54-56°C, and wherein the second stem comprises 4 base pairs having a sequence 5'-CACG-3'.

IN THE SPECIFICATION

Please replace the paragraph beginning on page 4, line 5, with the following paragraph.

a2

FIG. 1A, 1B, 1C Schematic of experimental design. (A) 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. (B) 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.* (C) 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.

37

Q

Please replace the paragraph beginning on page 14, line 29 with the following paragraph.

**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 (33,34) were synthesized by Midland Scientific and other

oligonucleotides were synthesized by Gene Link (Thornwood, NY). 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.

**SEQUENCE LISTING**

Please enter the enclosed paper copy of the Sequence Listing after the claims. A computer readable copy of the Sequence Listing is also enclosed herewith to comply with 37 § CFR 1.821(e). The content of the paper and computer readable copy of the



Sequence Listing, submitted in accordance with 37 CFR § 1.821 (c) and (e), respectively, are identical. The submitted Sequence Listing, filed in accordance with 37 CFR § 1.821 (g) herein does not include new matter.

#### REMARKS

##### The Invention

The invention is directed to a method for determining the ratio of a selected genetic sequence in a population of genetic sequences. Nucleic acid template molecules in a biological sample are diluted to form a set comprising a plurality of assay samples. The diluted nucleic acid template molecules are amplified to form a population of amplified molecules in the assay samples of the set. The amplified molecules 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. The first number and the second number are compared to ascertain a ratio that reflects the composition of the biological sample (claim 1).

The invention is also drawn to a method for determining the ratio of a selected genetic sequence in a population of genetic sequences. Template molecules within a set which comprises 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

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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 (claim 38).

The invention is also drawn to molecular beacon probes. The molecular beacon probe 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 and has a  $T_m$  of 50-51°C. The stem consists of 4 base pairs and has a sequence 5'-CACG-3' (claim 33). The loop of the molecular beacon probe may alternatively consist of 19-20 base pairs and have a  $T_m$  of 54-56°C (claim 36).

The invention also is drawn to a pair of molecular beacon probes comprising a first and second probe. This first probe comprises an oligonucleotide with a stem-loop structure having a photoluminescent dye at one of the 5' and 3' ends and a quenching agent at the opposite 5' or 3' end. The loop consists of 16 base pairs and has a  $T_m$  of 50-51°C. The stem consists of 4 base pairs and has a sequence 5'-CACG-3'. The second probe comprises an oligonucleotide that has a stem-loop structure having a photoluminescent dye at one of the 5' and 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 and has a sequence 5'CACG-3' (claim 37).

#### Information Disclosure Statement

The Office Action asserts that the listing of references in the specification is not a

proper information disclosure statement (IDS). The listing of references in the specification is not intended as the IDS for the application. Applicants have made two submissions on Form PTO-1449, in compliance with 37 CFR 1.98(b) on December 15, 2000 and March 7, 2001. A copy of each IDS submitted is attached along with the postcard receipts, at Tabs A and B. Clearly the PTO received at least one sheet of PTO-1449, as this has been returned to applicant, albeit entirely crossed out. No explanation is provided for the failure to consider the references. A new set of references is included in case these were lost in PTO handling. Li et al. is not included with this response, but will be sent in a separate mailing. Applicants recognize that the two lists of references are almost identical but for the Brown patent which was only listed on the March 7, 2001 submission. Applicants request an initialed copy of the PTO-1449 indicating consideration of each reference.

#### Objections to the Specification

The Office Action has objected to the specification for reciting "Figure 1" in the Brief Description of the Drawings, while no Figure 1 exists in the drawings. The specification has been amended to properly recite Figure 1A, 1B, 1C in the Brief Description of the Drawings.

The specification was further objected to for improper disclosure of nucleotide sequences. The sequences referenced in the Office Action (at page 14, lines 30 and 31, as well as sequences not referenced in the Office Action at page 15, lines 1, 2, 4, and 13 of the specification) were entered into a Sequence Listing as they appear in the

application, and thus contain no new matter. A paper and computer readable form of the Sequence Listing are submitted with this amendment.

The Rejection of Claims 1-64 under 35 U.S.C. § 112

Claims 1-64 have been 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 the applicant regards as his invention.

A. The rejected claims are allegedly incomplete for omitting essential steps. The Office Action identifies the omitted steps as "serially diluting to form a set of assay samples and testing by PCR." (Page 4, lines 3-4.) Applicants respectfully traverse.

Claim 1 recites both a diluting step and an amplifying step at lines 3 and 5, respectively. Claim 38 recites an amplifying step at line 3. Thus the only step that could possibly be missing is diluting in claim 38. However, this step is neither essential nor required. Claim 38 requires a certain concentration of template which may, but need not, be achieved by dilution. If samples are initially sufficiently dilute, no dilution is required. Thus dilution is not a necessary step.

The Office Action points to the specification at page 13, lines 17-19, to demonstrate that claims 1-32 and 38-64 omit the essential steps of serially diluting and testing via PCR. The citation is to example 1. The examples, however, are provided "for purposes of illustration only, and are not intended to limit the scope of the invention." (Page 13, lines 6-7.) Nothing in the example indicates that dilution is essential, and as discussed above, it is not.

B. The Office Action asserts that the claims omit linear amplification by PCR, which is allegedly a critical step of the invention. The PTO supports this assertion by citing the specification at page 14, line 18 where an example of sample analysis is disclosed in which linear amplification is used to enhance the signal provided by molecular beacon probes. Applicants respectfully traverse.

Claim 1 recites:

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.

Claim 38 recites:

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.

In each claim, the amplified molecules in the assay samples of the set are analyzed, but a particular analysis method is not required.

Linear amplification can be performed as part of the step of analyzing but it need not be. The specification teaches that: "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." (Emphasis added, page 12, lines 29-31.)

Since linear amplification was taught to enhance the signal of molecular beacon probes,

which are not essential, clearly linear amplification is not essential either. Therefore, the claimed method does not require linear PCR.

Nothing in the specification indicates that linear amplification by PCR is essential. Rather, linear amplification by PCR is disclosed as an enhancement to the analysis step when molecular beacon (MB) probes are used. The specification states, "fluorescent signals obtained could be considerably enhanced if several cycles of asymmetric, linear amplification were performed in the presence of the MB probes." (Page 19, lines 9-11.) Thus linear amplification is not essential to the method of the invention.

C. The Office Action asserts that the use of the term "consists" is confusing because "[i]t cannot be determined whether the claim intends open or closed language for the limitation of the sequence. Proper Markush language is required." (Page 4, lines 14-15.) Applicant's respectfully traverse.

Each of claims 33, 36, and 37 recite "the stem consists of 4 base pairs having a sequence 5'-CACG-3'." "When the phrase 'consists of' appears in a clause of the body of a claim, rather than immediately following the preamble, it limits only the element set forth in that clause." *Manesmann Demag Corp. v. Engineered Metal Products Co.*, 793 F.2d 1279, 230 USPQ 45 (Fed. Cir. 1986). Therefore, the term "consists" is closed. The stem contains the four recited base pairs 5'-CACG-3' and no others. No Markush group is present in claims 33, 36, and 37.

D. The Office Action asserts that "[c]laim 2 is confusing because it is unclear as to whether each sample of the fraction of one out ten (sic) are to contain N molecules."

(Page 4, lines 16-17.) Applicant's respectfully traverse.

The claim recites, "at least one-tenth of the assay samples in the set comprise a number (N) of molecules." (Claim 2, lines 2-3.) The claim positively recites that at least one out of ten of the assay samples in the set have a number (N) molecules. The claim is not confusing or unclear. The language of the claim affirmatively answers the question of the Office Action. Each of the 1/10 fraction of samples comprise a number (N) molecules. N is defined so that 1/N is larger than the ratio of selected genetic sequences to total genetic sequences. Thus all of the 1/10 samples need not have the same number of molecules, but a number that fits the definition. Nonetheless, if samples are formed by dilution, as in claim 2, the samples should have roughly identical numbers of molecules.

Withdrawal of the 35 U.S.C. §112 rejection of claims 1-64 is respectfully requested as all claims are clear and definite.

Rejection of claims 1 and 3-32 under 35 U.S.C. §103(a)

Lapidus (U.S. 5,928,870) and Ruano (P.N.A.S., vol. 87, pp. 6296-6300, August 1990) in combination are cited as teaching the invention of claims 1, 3, 4-11, 14-16, and 19-32. Tyagi (U.S. 5,925,517) is further combined to allegedly teach the invention of claims 12, 13, 17, and 18. These rejections are respectfully traversed.

It is axiomatic that all elements of a claim must be taught or suggested by the prior art for a *prima facie* case of obviousness to be proper. MPEP §2143. The present rejection fails to fulfill this "all elements" rule and thus fails to present a *prima facie* case.

Claim 1 requires four steps: diluting, amplifying, analyzing, and comparing.

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Neither Lapidus nor Ruano teach the step of analyzing or the step of comparing as specified in claim 1. Claim 1, steps 3 and 4, recite:

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 biological sample.

Emphasis added. The Office Action fails to point to any portion in either Lapidus or Ruano which teach these two steps. Lapidus does not teach determining a number of assay samples containing genetic sequences. Lapidus instead teaches determining concentration. The Office Action refers to this teaching of Lapidus as "enumerating number molecules of a target," citing col. 2, lines 58-66. This, however, is different from determining the number of assay samples containing a genetic sequence. Since the numbers of assay samples are not determined according to Lapidus, neither are the numbers compared, as required in step 4.

This difference leads to an advantage of the present invention over Lapidus. Digital amplification, as claimed, converts "the intrinsically exponential nature of PCR to a linear one." Specification at page 8, lines 17-18. Thus the present invention eliminates the quantitative bias which exponential amplification introduces into a nucleic acid sample. Since neither Lapidus nor Ruano teach these elements of the claims, the *prima facie* case must fail.

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Tyagi teaches molecular beacon probes. Tyagi is cited in combination with Lapidus and Ruano to allegedly render claims 12, 13, 17 and 18 obvious. (Claim 12 does not employ a molecular beacon probe at all, so its inclusion in this rejection is improper.)

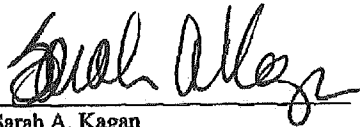
Like the primary references, Tyagi does not teach the element of "determining a first number of assay samples" nor of comparing the first and second numbers. Thus Tyagi does not remedy the defect of the primary references. Again, the *prima facie* case fails to teach all elements of the claimed invention and must therefore be withdrawn as improper.

A speedy allowance of all pending claims is respectfully requested.

Respectfully submitted,

Date: July 12, 2001

By:



Sarah A. Kagan  
Registration No. 32,141

BANNER & WITCOFF, LTD.  
1001 G STREET, NW  
WASHINGTON, DC 20001  
202-508-9100



**MARKED UP VERSION TO SHOW CHANGES MADE**

Replacement paragraph beginning on page 4, line 5.

FIG. 1A, 1B, 1C. Schematic of experimental design. (A) 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. (B) 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.* . (C) 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.

Replacement paragraph beginning on page 14, line 29.

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:

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5'-Cy3-CACGGGCCTGCTGAAAAATGACTGCGTG-Dabcyl-3' (SEQ ID NO: 4);  
MB-GREEN: 5'-Fluorescein-CACGGGAGCTGGTGGCGTAGCGTG-Dabcyl-3' (SEQ ID NO: 5). Molecular Beacons (33,34) were synthesized by Midland Scientific and other oligonucleotides were synthesized by Gene Link (Thornwood, NY). 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'-CATTATTTTATTATAAGGCCTGC-3' (SEQ ID NO: 6). Sequencing was performed using fluorescently-labeled ABI Big Dye terminators and an ABI 377 automated sequencer.

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Application No.: 09/613826

**NOTICE TO COMPLY WITH REQUIREMENTS FOR PATENT APPLICATIONS CONTAINING NUCLEOTIDE SEQUENCE AND/OR AMINO ACID SEQUENCE DISCLOSURES**

Applicant must file the items indicated below within the time period set the Office action to which the Notice is attached to avoid abandonment under 35 U.S.C. § 133 (extensions of time may be obtained under the provisions of 37 CFR 1.138(a)).

The nucleotide and/or amino acid sequence disclosure contained in this application does not comply with the requirements for such a disclosure as set forth in 37 C.F.R. 1.821 - 1.825 for the following reason(s):

- 1. This application clearly fails to comply with the requirements of 37 C.F.R. 1.821-1.825. Applicant's attention is directed to the final rulemaking notice published at 55 FR 18230 (May 1, 1990), and 1114 OG 29 (May 15, 1990). If the effective filing date is on or after July 1, 1998, see the final rulemaking notice published at 63 FR 29620 (June 1, 1998) and 1211 OG 82 (June 23, 1998).
- 2. This application does not contain, as a separate part of the disclosure on paper copy, a "Sequence Listing" as required by 37 C.F.R. 1.821(c).
- 3. A copy of the "Sequence Listing" in computer readable form has not been submitted as required by 37 C.F.R. 1.821(e).
- 4. A copy of the "Sequence Listing" in computer readable form has been submitted. However, the content of the computer readable form does not comply with the requirements of 37 C.F.R. 1.822 and/or 1.823, as indicated on the attached copy of the marked-up "Raw Sequence Listing."
- 5. The computer readable form that has been filed with this application has been found to be damaged and/or unreadable as indicated on the attached CRF Diskette Problem Report. A Substitute computer readable form must be submitted as required by 37 C.F.R. 1.825(d).
- 6. The paper copy of the "Sequence Listing" is not the same as the computer readable form of the "Sequence Listing" as required by 37 C.F.R. 1.821(e).
- 7. Other: \_\_\_\_\_

**Applicant Must Provide:**

- An initial or substitute computer readable form (CRF) copy of the "Sequence Listing".
- An initial or substitute paper copy of the "Sequence Listing", as well as an amendment directing its entry into the specification.
- A statement that the content of the paper and computer readable copies are the same and, where applicable, include no new matter, as required by 37 C.F.R. 1.821(e) or 1.821(f) or 1.821(g) or 1.825(b) or 1.825(d).

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PATENT APPLICATION

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

|                                |   |                        |
|--------------------------------|---|------------------------|
| In re application of:          | ) |                        |
| Bert Vogelstein, <i>et al.</i> | ) | Group Art No. 1632     |
| Serial No.: 09/613,826         | ) | Examiner: TBA          |
| Filed: July 11, 2000           | ) | Docket No. 01107.00031 |
| For: DIGITAL AMPLIFICATION     | ) |                        |

INFORMATION DISCLOSURE STATEMENT

Assistant Commissioner for Patents  
Washington, D.C. 20231

Sir:

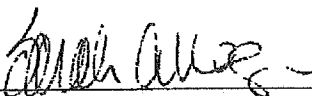
Pursuant to 37 C.F.R. §1.56 and in compliance with 37 C.F.R. §1.97, Applicants submit herewith a Form PTO-1449 identifying information for consideration by the Examiner. A copy of each of the items of information is enclosed.

Applicants do not waive any rights to take appropriate action to establish patentability over the listed documents should they be applied as a reference against the claims of the present application.

Consideration of the cited information and making the same of record in the prosecution of the above-noted application are respectfully requested. Should the Patent and Trademark Office determine that a fee is required, please charge our Deposit Account No. 19-0733.

Respectfully submitted,

BANNER & WITCOFF, LTD.

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

1001 G Street, N.W.  
 Washington, D.C. 20001-4597  
 (202) 508-9100  
 Dated: 03-05-01

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|---------------------------------------------------------------------------------------------------------------------------------------|---------------------------------------|--------------------------------|
| PTO-1449 (Modified)<br>U.S. DEPARTMENT OF COMMERCE<br>PATENT AND TRADEMARK OFFICE<br>INFORMATION DISCLOSURE STATEMENT<br>BY APPLICANT | ATTY. DOCKET NO.<br>01107.00031       | SERIAL NUMBER<br>TBA 09/613826 |
|                                                                                                                                       | APPLICANTS<br>Bert Vogelstein, et al. |                                |
|                                                                                                                                       | FILING DATE<br>July 11, 2000          | GROUP ART UNIT<br>TBA 1637     |
|                                                                                                                                       |                                       |                                |

U.S. PATENT DOCUMENTS

| EXAMINER INITIAL | DOCUMENT NUMBER | DATE    | NAME            | CLASS | SUB CLASS | FILING DATE |
|------------------|-----------------|---------|-----------------|-------|-----------|-------------|
| JS               | 5,670,825       | 9/1997  | Lapidus, et al. |       |           |             |
|                  | 5,928,870       | 7/1999  | Lapidus, et al. |       |           |             |
|                  | 6,020,137       | 2/2000  | Lapidus, et al. |       |           |             |
| JS               | 6,143,496       | 11/2000 | Brown, et al.   |       |           |             |

OTHER DOCUMENTS (Including Author, Title, Date, Pertinent Pages, Etc.)

|    |                                                                                                                                                                                                                                                                                                 |
|----|-------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------|
| JS | Daven G. MONCKTON, et al., "Minisatellite "Isoclele" Discrimination in Pseudohomozygotes by Single Molecule PCR and Variant Repeat Mapping", <i>Genomics</i> 11, pp. 465-467, 1991                                                                                                              |
|    | Gualberto RUANO, et al., "Haplotype of Multiple Polymorphisms Resolved by Enzymatic Amplification of Single DNA Molecules", <i>Proc. National Science USA</i> , 1990                                                                                                                            |
|    | W. NAVIDI, et al., "Using PCR in Preimplantation Genetic Disease Diagnosis", <i>Human Reproduction</i> , Vol. 6, No. 6, pp. 836-849, 1991                                                                                                                                                       |
|    | Honghua LI, et al., "Amplification and Analysis of DNA Sequences in Single Human Sperm and Diploid Cells", <i>Nature</i> , Vol. 335, September 29, 1988                                                                                                                                         |
|    | Ramon PARSONS, et al., "Mismatch Repair Deficiency in Phenotypically Normal Human Cells", <i>Science</i> , Vol. 268, May 5, 1995                                                                                                                                                                |
|    | Lin ZHANG, et al., "Whole Genome Amplification from a Single Cell: Implications for Genetic Analysis", <i>Proc. National Science USA</i> , Vol. 89, pp. 5847-5851, July 1992                                                                                                                    |
|    | David SIDRANSKY, et al., "Clonal Expansion of p53 Mutant Cells is Associated with Brain Tumour Progression", <i>Nature</i> , February 27, 1992                                                                                                                                                  |
|    | Alec J. Jeffreys, et al., "Mutation Processes at Human Minisatellites", <i>Electrophoresis</i> , pp. 1577-1585, 1995                                                                                                                                                                            |
|    | 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", <i>Forensic Science International</i> , Vol. 66, pp. 129-141, 1994 |
|    | Paul M. LIZARDI, et al., "Mutation Detection and Single-Molecule Counting Using Isothermal Rolling-Circle Amplification", <i>Nature Genetics</i> , Vol. 19, July 1998                                                                                                                           |
|    | W. NAVIDI, et al., "Using PCR in Preimplantation Genetic Disease Diagnosis", <i>Human Reproduction</i> , Vol. 6, 1991                                                                                                                                                                           |
| JS | Honghua LI, et al., "Amplification and Analysis of DNA Sequences in Single Human Sperm and Diploid Cells" <i>Nature</i> , Vol. 335, September 29, 1988                                                                                                                                          |

EXAMINER Jeffrey Liu DATE CONSIDERED 8/23/02

EXAMINER: Initial citation if reference was considered. Draw line through citation if not in conformance to MPEP 609 and not considered. Include copy of this form with next communication to applicant.



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Patent/Design

PATENT  DESIGN

HAND CARRY Group/Section \_\_\_\_\_ Bldg \_\_\_\_\_ Rm \_\_\_\_\_  
Serial No. 09/613526 B&W # 0110700031 Atty/Sec SJK/a.m.c. Date 12/15/00  
Inventor Robertson Client JHU  
Title Digital Annotation

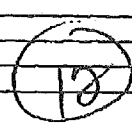
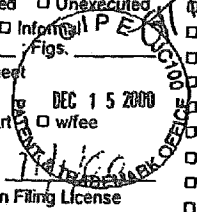
- The following has been received in the U.S. Patent and Trademark Office on the date stamped hereon:
- total pp Spec., including : # of Claims \_\_\_\_\_  Claim for Priority w/Priority Doc \_\_\_\_\_  
(# of independent claims \_\_\_\_\_);  Abstract \_\_\_\_\_ Country, Appl # and Date \_\_\_\_\_
  - Foreign Priority on \_\_\_\_\_  Petition for Extension til \_\_\_\_\_  
Country, Appl # and Date \_\_\_\_\_
  - Priority on U.S. Prov. \_\_\_\_\_ B&W# \_\_\_\_\_  Amendment  Response : OA dtd \_\_\_\_\_
  - Application :  CIP  Continuation  Divisional  Request for Approval of Drawing Changes
  - Parent Ser. No. \_\_\_\_\_ B&W# \_\_\_\_\_  CPA Request  w/Ext of Time : OA dtd \_\_\_\_\_
  - Provisional App \_\_\_\_\_ pp Spec/Clms; Cover Sht.  Notice of Appeal & Fee
  - Brief :  Appeal & Fee  Reply
  - Request for Oral Hearing

Patent/Design

PATENT  DESIGN

HAND CARRY Group/Section \_\_\_\_\_ Bldg \_\_\_\_\_ Rm \_\_\_\_\_  
Serial No. 09/613526 B&W # 0110700031 Atty/Sec SJK/a.m.c. Date 12/15/00  
Inventor Robertson Client JHU  
Title Digital Annotation

- The following has been received in the U.S. Patent and Trademark Office on the date stamped hereon:
- total pp Spec., including : # of Claims \_\_\_\_\_  Claim for Priority w/Priority Doc \_\_\_\_\_  
(# of independent claims \_\_\_\_\_);  Abstract \_\_\_\_\_ Country, Appl # and Date \_\_\_\_\_
  - Foreign Priority on \_\_\_\_\_  Petition for Extension til \_\_\_\_\_  
Country, Appl # and Date \_\_\_\_\_
  - Priority on U.S. Prov. \_\_\_\_\_ B&W# \_\_\_\_\_  Amendment  Response : OA dtd \_\_\_\_\_
  - Application :  CIP  Continuation  Divisional  Request for Approval of Drawing Changes
  - Parent Ser. No. \_\_\_\_\_ B&W# \_\_\_\_\_  CPA Request  w/Ext of Time : OA dtd \_\_\_\_\_
  - Provisional App \_\_\_\_\_ pp Spec/Clms; Cover Sht.  Notice of Appeal & Fee
  - Declaration/PoA :  Executed  Unexecuted  Brief :  Appeal & Fee  Reply
  - Drawings :  Formal  Informal  Request for Oral Hearing
  - # of distinct sheets \_\_\_\_\_ Figs. \_\_\_\_\_  Issue Fee : Allowance dtd \_\_\_\_\_
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|----------------------------------------------------------------------------------------------------|-----------------------------|-------------------------------|-----------------------------------------------------------------------|
| <b>TRANSMITTAL FORM</b><br><small>(to be used for all correspondence after initial filing)</small> | <b>Application Number</b>   | 09/613,826                    | <b>RECEIVED</b><br><b>JUL 17 2001</b><br><b>TECH CENTER 1000/2900</b> |
|                                                                                                    | <b>Filing Date</b>          | July 11, 2000                 |                                                                       |
|                                                                                                    | <b>First Named Inventor</b> | VOGELSTEIN, et al.            |                                                                       |
|                                                                                                    | <b>Group Art Unit</b>       | 1656                          |                                                                       |
|                                                                                                    | <b>Examiner Name</b>        | J. SIEW                       |                                                                       |
| <b>Total Number of Pages in This Submission</b>                                                    | 27                          | <b>Attorney Docket Number</b> | 01107.00031                                                           |

| ENCLOSURES (check all that apply)                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                    |                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                  |                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                |
|------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------|------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------|--------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------|
| <input type="checkbox"/> Fee Transmittal Form<br><br><input type="checkbox"/> Fee Attached<br><br><input checked="" type="checkbox"/> Amendment / Response<br><br><input type="checkbox"/> After Final<br><br><input type="checkbox"/> Affidavits/declaration(s)<br><br><input type="checkbox"/> Extension of Time Request<br><br><input type="checkbox"/> Express Abandonment Request<br><br><input type="checkbox"/> Information Disclosure Statement<br><br><input type="checkbox"/> Certified Copy of Priority Document(s)<br><br><input type="checkbox"/> Response to Missing Parts/ Incomplete Application<br><br><input type="checkbox"/> Response to Missing Parts under 37 CFR 1.52 or 1.53 | <input type="checkbox"/> Assignment Papers (for an Application)<br><br><input type="checkbox"/> Drawing(s)<br><br><input type="checkbox"/> Licensing-related Papers<br><br><input type="checkbox"/> Petition Routing Slip (PTO/SB/88) and Accompanying Petition<br><br><input type="checkbox"/> Petition to Convert to a Provisional Application<br><br><input type="checkbox"/> Power of Attorney, Revocation Change of Correspondence Address<br><br><input type="checkbox"/> Terminal Disclaimer<br><br><input type="checkbox"/> Request for Refund<br><br><input type="checkbox"/> CD, Number of CD(s) _____ | <input type="checkbox"/> After Allowance Communication to Group<br><br><input type="checkbox"/> Appeal Communication to Board of Appeals and Interferences<br><br><input type="checkbox"/> Appeal Communication to Group (Appeal Notice, Brief, Reply Brief)<br><br><input type="checkbox"/> Proprietary Information<br><br><input type="checkbox"/> Status Letter<br><br><input checked="" type="checkbox"/> Other Enclosure(s) (please identify below):<br>Sequence Listing; Diskette Containing Sequence Listing;<br>Copy of Notice to Comply;<br>copies of 2 previously submitted IDS's; copies of references cited on IDS |
| <b>Remarks</b><br><i>Pages of references are in addition to Total Number of pages listed</i>                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                         |                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                  |                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                |

| SIGNATURE OF APPLICANT, ATTORNEY, OR AGENT |                                   |
|--------------------------------------------|-----------------------------------|
| Firm or Individual name                    | Sarah A. Kagan<br>Reg. No. 32,141 |
| Signature                                  |                                   |
| Date                                       | July 12, 2001                     |

| CERTIFICATE OF MAILING                                                                                                                                                                                                             |       |      |
|------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------|-------|------|
| I hereby certify that this correspondence is being deposited with the United States Postal Service as first class mail in an envelope addressed to; Assistant Commissioner for Patents, Washington, D.C. 20231 on this date: _____ |       |      |
| Typed or printed name                                                                                                                                                                                                              | _____ |      |
| Signature                                                                                                                                                                                                                          | _____ | Date |

Burden Hour Statement: This form is estimated to take 0.2 hours to complete. Time will vary depending upon the needs of the individual case. Any comments on the amount of time you are required to complete this form should be sent to the Chief Information Officer, U.S. Patent and Trademark Office, Washington, DC 20231. DO NOT SEND FEES OR COMPLETED FORMS TO THIS ADDRESS. SEND TO: Assistant Commissioner for Patents, Washington, DC 20231.

U



SEQUENCE LISTING

<110> Vogelstein, Bert  
Kinzler, Kenneth W.

<120> DIGITAL AMPLIFICATION

<130> 01107.00031

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<141> 2000-07-11

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<151> 1999-08-02

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PATENT

#9  
MB  
07/23/01

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re Application of

Bert Vogelstein, et. al.

Serial No. 09/613,826

Filing Date: July 11, 2000

For: **DIGITAL AMPLIFICATION**



)  
) Group Art Unit: 1656  
)  
) Examiner: I Siew  
)  
)  
) Docket No. 01107.00031

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JUL 19 2001  
TECH CENTER 1600/2900

SUPPLEMENTAL SUBMISSION

Assistant Commissioner for Patents  
Washington, D.C. 20231

Sir:

In applicants' response to the Office Action mailed July 12, 2001 copies of two previously filed Information Disclosure Statements (IDS) were supplied. A new set of references was also included in case the original set of references was lost in PTO handling. A copy of Li, et al. (Nature, 1988, (335):414-417) however was missing. The reference is enclosed herewith.

No fee is believed due. If any additional fee is due please change our Deposit Account No. 19-0733.

Respectfully submitted,

Date: July 17, 2001

By: Michelle Holmes-Son  
Michelle L. Holmes-Son  
Registration No. 47,660

BANNER & WITCOFF, LTD.  
1001 G STREET, NW  
WASHINGTON, DC 20001  
202-508-9100

Please type a plus sign (+) inside this box →

PTO/SB/21 (08-00)  
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**TRANSMITTAL FORM**

(to be used for all correspondence after initial filing)

|                        |                   |
|------------------------|-------------------|
| Application Number     | 09/613,826        |
| Filing Date            | July 11, 2000     |
| First Named Inventor   | VOGELSTEIN et al. |
| Group Art Unit         | 1656              |
| Examiner Name          | J. SIEW           |
| Attorney Docket Number | 01107.00031       |

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TECH CENTER 1600/290

|                                          |   |
|------------------------------------------|---|
| Total Number of Pages in This Submission | 6 |
|------------------------------------------|---|

ENCLOSURES (check all that apply)

|                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                   |                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                  |                                                                                                                                                                                                                                                                                                                                                                                                                                                                                       |
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| <input type="checkbox"/> Fee Transmittal Form<br><input type="checkbox"/> Fee Attached<br><input type="checkbox"/> Amendment / Response<br><input type="checkbox"/> After Final<br><input type="checkbox"/> Affidavits/declaration(s)<br><input type="checkbox"/> Extension of Time Request<br><input type="checkbox"/> Express Abandonment Request<br><input type="checkbox"/> Information Disclosure Statement<br><input type="checkbox"/> Certified Copy of Priority Document(s)<br><input type="checkbox"/> Response to Missing Parts/ Incomplete Application<br><input type="checkbox"/> Response to Missing Parts under 37 CFR 1.52 or 1.53 | <input type="checkbox"/> Assignment Papers (for an Application)<br><input type="checkbox"/> Drawing(s)<br><input type="checkbox"/> Licensing-related Papers<br><input type="checkbox"/> Petition Routing Slip (PTO/SB/89) and Accompanying Petition<br><input type="checkbox"/> Petition to Convert to a Provisional Application<br><input type="checkbox"/> Power of Attorney, Revocation Change of Correspondence Address<br><input type="checkbox"/> Terminal Disclaimer<br><input type="checkbox"/> Request for Refund<br><input type="checkbox"/> CD, Number of CD(s) _____ | <input type="checkbox"/> After Allowance Communication to Group<br><input type="checkbox"/> Appeal Communication to Board of Appeals and Interferences<br><input type="checkbox"/> Appeal Communication to Group (Appeal Notice, Brief, Reply Brief)<br><input type="checkbox"/> Proprietary Information<br><input type="checkbox"/> Status Letter<br><input checked="" type="checkbox"/> Other Enclosure(s) (please identify below):<br>Supplemental Submission<br>Copy of Reference |
| Remarks                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                           |                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                  |                                                                                                                                                                                                                                                                                                                                                                                                                                                                                       |

SIGNATURE OF APPLICANT, ATTORNEY, OR AGENT

|                         |                                        |
|-------------------------|----------------------------------------|
| Firm or Individual name | Michelle Holmes-Son<br>Reg. No. 47,680 |
| Signature               | <i>Michelle Holmes-Son</i>             |
| Date                    | July 17, 2001                          |

CERTIFICATE OF MAILING

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|-----------------------|-------|
| Typed or printed name | _____ |
| Signature             | _____ |
| Date                  | _____ |

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Patent and Trademark Office**

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Washington, D.C. 20231

10

| APPLICATION NO. | FILING DATE | FIRST NAMED INVENTOR | ATTORNEY DOCKET NO. |
|-----------------|-------------|----------------------|---------------------|
|-----------------|-------------|----------------------|---------------------|

|            |          |            |              |
|------------|----------|------------|--------------|
| 09/613,826 | 07/11/00 | VOGELSTEIN | D 01107,0003 |
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1001 G STREET N W  
SUITE 1100  
WASHINGTON DC 20001

RM12/0920

EXAMINER

| ART UNIT | PAPER NUMBER |
|----------|--------------|
|----------|--------------|

1656  
DATE MAILED: 09/20/01

10

Please find below and/or attached an Office communication concerning this application or proceeding.

Commissioner of Patents and Trademarks



|                              |                 |                   |  |
|------------------------------|-----------------|-------------------|--|
| <b>Office Action Summary</b> | Application No. | Applicant(s)      |  |
|                              | 09/313,826      | VOGELSTEIN ET AL. |  |
|                              | Examiner        | Art Unit          |  |
|                              | Jeffrey Siew    | 1656              |  |

*-- 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) 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 the period for reply specified above is less than thirty (30) days, a reply within the statutory minimum of thirty (30) days will be considered timely.
- 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 12 July 2001.

2a)  This action is FINAL.                      2b)  This action is non-final.

3)  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**

4)  Claim(s) 1-69 is/are pending in the application.

    4a) Of the above claim(s) \_\_\_\_\_ is/are withdrawn from consideration.

5)  Claim(s) 1-64 is/are allowed.

6)  Claim(s) 65-69 is/are rejected.

7)  Claim(s) \_\_\_\_\_ is/are objected to.

8)  Claim(s) \_\_\_\_\_ are subject to restriction and/or election requirement.

**Application Papers**

9)  The specification is objected to by the Examiner.

10)  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).

11)  The proposed drawing correction filed on \_\_\_\_\_ is: a)  approved b)  disapproved by the Examiner.

If approved, corrected drawings are required in reply to this Office action.

12)  The oath or declaration is objected to by the Examiner.

**Priority under 35 U.S.C. §§ 119 and 120**

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.

14)  Acknowledgment is made of a claim for domestic priority under 35 U.S.C. § 119(e) (to a provisional application).

a)  The translation of the foreign language provisional application has been received.

15)  Acknowledgment is made of a claim for domestic priority under 35 U.S.C. §§ 120 and/or 121.

**Attachment(s)**

|                                                                                                            |                                                                             |
|------------------------------------------------------------------------------------------------------------|-----------------------------------------------------------------------------|
| 1) <input type="checkbox"/> Notice of References Cited (PTO-892)                                           | 4) <input type="checkbox"/> Interview Summary (PTO-413) Paper No(s). _____  |
| 2) <input type="checkbox"/> Notice of Draftsperson's Patent Drawing Review (PTO-948)                       | 5) <input type="checkbox"/> Notice of Informal Patent Application (PTO-152) |
| 3) <input checked="" type="checkbox"/> Information Disclosure Statement(s) (PTO-1449) Paper No(s) <u>3</u> | 6) <input type="checkbox"/> Other: _____                                    |

**DETAILED ACTION**

*Information Disclosure Statement*

1. The IDS filed 12/15/00 was one page and IDS filed March 7, 2001 was one page. Both were signed and intended to be mailed to applicant. Apparently the IDS of 12/15/00 was only received. The references on this IDS were crossed out because they are duplicates of references on IDS March 7, 2001. Moreover, all the references in newly submitted IDS July 12, 2001 were contained in the IDS of March 7, 2001. It is unclear as to the purpose applicant's resubmission of these references but as the office has reviewed the references per IDS March 7, 2001 and signed the PTO-1449 it is deemed adequately considered. A copy of signed IDS March 7, 2001 will be resent with this mailing.

**THE FOLLOWING IS A NEW GROUND OF REJECTION NECESSITATED BY THE AMENDMENT**

*Claim Rejections - 35 USC § 112*

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

The specification shall contain a written description of the invention, and of the manner and process of making and using it, in such full, clear, concise, and exact terms as to enable any person skilled in the art to which it pertains, or with which it is most nearly connected, to make and use the same and shall set forth the best mode contemplated by the inventor of carrying out his invention.

Claims 65-69 are rejected under 35 U.S.C. 112, first paragraph, as containing subject matter which was not described in the specification in such a way as to reasonably convey to one

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Page 3

Art Unit: 1656

skilled in the relevant art that the inventor(s), at the time the application was filed, had possession of the claimed invention. The specification describes molecular probes that are consisting of 16 base pairs with a T<sub>m</sub> of 50-51°C and a stem consisting of 4 base pairs or one with a loop consisting of 19-20 base pairs and T<sub>m</sub> of 54-56°C and stem consisting of 4 base pairs. The specification lacks support for molecular beacon that has a loop greater than 16 base pairs with T<sub>m</sub> of 50-51°C and stem comprising 4 base pairs nor a molecular beacon that a loop comprising 19-20 base pairs and T<sub>m</sub> of 54-56°C and stem comprising of 4 base pairs.

3. 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 68 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.

A) The term comprising 19-20 renders claim 68 unclear. As the term is open it is unclear as to whether the loop is to be greater than 19 or greater than 20 base pairs.

***Claim Rejections - 35 USC § 102***

4. 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 --

(e) the invention was described in a patent granted on an application for patent by another filed in the United States before the invention thereof by the applicant for patent, or on an international application by another who has fulfilled the requirements of paragraphs (1), (2), and (4) of section 371(c) of this title before the invention thereof by the applicant for patent.

Claim 65-67 are rejected under 35 U.S.C. 102(e) as being anticipated by Tyagi et al (US5,925,517 March 14, 2000).

Tyagi et al who teach a molecular beacon with a stem comprising CACG (see col. 11 probe 3) but with a loop of Tm 50  $Tm = [(A+T) \times 2C + (G+C) \times 4C]$  (see col. 12 SEQ ID NO:3).

Claims 66 & 67 refer to a property that is drawn to the intended use of the probe.

Upon recalculation of the loop for Tyagi et al's probe 3, it appears that the Tm is within the claimed range. However, in referring to original claim 33 probe 3 does not have the limitation of stem of only 4 base pairs.

#### SUMMARY

5. Claims 33-37 are allowable. There is no prior art that teach or suggest a molecular beacon probe that has a loop consisting of 16 base pairs and having a Tm of 50-51C and the stem consisting of CACG sequence. The closest prior art is Tyagi et al (US6,037,130) teach molecular beacon with a stem comprising CACG (see col. 11 probe 3) but with a loop of Tm 50C. Moreover, the prior art has been focused on the Tm of the stem which relates to the functioning of the opening and closing of the hairpin during hybridization.

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Art Unit: 1656

Page 5

Claims 1-32 & 38-64 is allowable. There is no prior art that teach or suggest diluting a nucleic acid template in a sample to a plurality of sample and amplifying the template molecule in the samples and analyzing amplified molecules to determine the first number of samples containing the selected genetic sequence and second number assay samples which contain a reference genetic sequence and comparing the two numbers. Moreover, there is no prior art that teach or suggest that one tenth or one fiftieth of samples in a set comprise N molecules such that  $1/N$  is larger than the ratio of selected genetic sequence to total genetic sequences required for the step of analyzing to determine presence of selected genetic sequence. The closest prior art is Lapidus et al who teach a reference and target nucleic acid amplification and concentration determination. However, his determination of concentration is within a sample and they do not teach or suggest a dilution.

#### 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,

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Art Unit: 1656

Page 6

however, will the statutory period for reply expire later than SIX MONTHS from the date of this final action.

6. Any inquiry concerning this communication or earlier communications from the examiner should be directed to Jeffrey Siew whose telephone number is (703) 305-3886 and whose e-mail address is Jeffrey.Siew@uspto.gov. However, the office cannot guarantee security through the e-mail system nor should official papers be transmitted through this route. The examiner is on flex-time schedule and can best be reached on weekdays from 6:30 a.m. to 3 p.m. If attempts to reach the examiner are unsuccessful, the examiner's supervisor, Gary Jones, can be reached on (703)-308-1152.

Any inquiry of a general nature or relating to the status of this application or proceeding should be directed to the receptionist for Technology Center 1600 whose telephone number is (703) 308-0196.

Papers related to this application may be submitted to Group 1600 by facsimile transmission. Papers should be faxed to Group 1600 via the PTO Fax Center located in Crystal Mall 1. The faxing of such papers must conform with the notice published in the Official Gazette, 1096 OG 30 (November 15, 1989). The CM1 Center numbers for Group 1600 are Voice (703) 308-3290 and Fax (703) 308-4556 or (703) 308-4242.

  
Jeffrey Siew

September 20, 2001

|                          |                                      |                                          |  |
|--------------------------|--------------------------------------|------------------------------------------|--|
| <b>Interview Summary</b> | <b>Application No.</b><br>09/613,826 | <b>Applicant(s)</b><br>VOGELSTEIN ET AL. |  |
|                          | <b>Examiner</b><br>Jeffrey Siew      | <b>Art Unit</b><br>1656                  |  |

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

(1) Jeffrey Siew (3) \_\_\_\_\_

(2) Michelle Holmes-Son (4) \_\_\_\_\_

Date of Interview: \_\_\_\_\_

Type: a)  Telephonic b)  Video Conference  
c)  Personal [copy given to: 1)  applicant 2)  applicant's representative]

Exhibit shown or demonstration conducted: d)  Yes e)  No.  
If Yes, brief description: \_\_\_\_\_

Claim(s) discussed: None

Identification of prior art discussed: \_\_\_\_\_

Agreement with respect to the claims f)  was reached. g)  was not reached. h)  N/A.


Substance of Interview including description of the general nature of what was agreed to if an agreement was reached, or any other comments: applicant did not receive signed IDS of 1/23/02, office submitted signed already considered IDS

(A fuller description, if necessary, and a copy of the amendments which the examiner agreed would render the claims allowable, if available, must be attached. Also, where no copy of the amendments that would render the claims allowable is available, a summary thereof must be attached.)

i)  It is not necessary for applicant to provide a separate record of the substance of the interview (if box is checked).

Unless the paragraph above has been checked, THE FORMAL WRITTEN REPLY TO THE LAST OFFICE ACTION MUST INCLUDE THE SUBSTANCE OF THE INTERVIEW. (See MPEP Section 713.04). If a reply to the last Office action has already been filed, APPLICANT IS GIVEN ONE MONTH FROM THIS INTERVIEW DATE TO FILE A STATEMENT OF THE SUBSTANCE OF THE INTERVIEW. See Summary of Record of Interview requirements on reverse side or on attached sheet.

Examiner Note: You must sign this form unless it is an Attachment to a signed Office action.

  
Examiner's signature, if required

J. Siew

OIPE

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4 Kinzler, Kenneth W.  
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## File History Report

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The following checked item(s) below of paper number \_\_\_\_\_ is/are missing from the United States Patent and Trademark Office's original copy of the file history. No additional information is available

- PTO 1449
- PTO 892
- PTO 948
- PTO 1474
- Assignment
- Cover page

Additional comments: \_\_\_\_\_

S. Siew

← Re-run

Page 1 of 3

1656  
# 12  
DMS  
10.301  
OIPE

RAW SEQUENCE LISTING  
PATENT APPLICATION: US/09/613,826

DATE: 09/20/2001  
TIME: 17:06:04

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SEP 28 2001

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3 <110> APPLICANT: Vogelstein, Bert
4   Kinzler, Kenneth W.
6 <120> TITLE OF INVENTION: DIGITAL AMPLIFICATION
8 <130> FILE REFERENCE: 01107.00031
10 <140> CURRENT APPLICATION NUMBER: 09/613,826
11 <141> CURRENT FILING DATE: 2000-07-11
13 <150> PRIOR APPLICATION NUMBER: US 60/146,792
14 <151> PRIOR FILING DATE: 1999-08-02
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9/20/01

VERIFICATION SUMMARY  
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DATE: 09/20/2001  
TIME: 17:06:05

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SEP 28 2001

TECH CENTER 1600/2900

STATISTICS SUMMARY                      DATE: 09/20/2001  
PATENT APPLICATION: US/09/613,826        TIME: 17:06:05

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Output Set: N:\CRF3\09202001\I613826.raw

Application Serial Number: US/09/613,826  
Alpha or Numeric: Numeric  
Application Class:  
Application File Date: 07-11-2000  
Art Unit: OIPE  
Software Application: PatentIn  
Total Number of Sequences: 6  
Total Nucleotides: 145  
Total Amino Acids: 0  
Number of Errors: 0  
Number of Warnings: 0  
Number of Corrections: 0

MESSAGE SUMMARY



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PATENT

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12-10-01  
N.E.

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

|                            |                          |
|----------------------------|--------------------------|
| In re Application of       | )                        |
| Bert Vogelstein, et. al.   | ) Group Art Unit: 1656   |
| Serial No. 09/613,826      | ) Examiner: J Siew       |
| Filing Date: July 11, 2000 | ) Box AF                 |
|                            | ) Docket No. 01107.00031 |

For: **DIGITAL AMPLIFICATION**

AMENDMENT AFTER FINAL REJECTION

Assistant Commissioner for Patents  
Washington, D.C. 20231

Sir:

In response to the Final Office Action mailed September 20, 2001, applicants request entry of the following amendments and request reconsideration of the claims.

Claims 1-69 are pending in the application. Claims 1-64 are allowed, and claims 65-69 are rejected. No fees are believed due to make this response filed timely. If any fee is due please change our Deposit Account No. 19-0733.

*Do not  
enter  
photo  
for*

IN THE CLAIMS

Please amend claims 33, 36-37, 65, and 68-69.

33. (Amended) A molecular beacon probe comprising:

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, wherein the loop consists of 16 bases, wherein the loop has a  $T_m$  of 50-51°C and the stem consists of 4 base pairs having a sequence 5'-CACG-3'.

36. (Amended) A molecular beacon probe comprising:

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, wherein the loop consists of 19-20 bases, wherein the loop has a  $T_m$  of 54-56°C and the stem consists of 4 base pairs having a sequence 5'-CACG-3'.

37. (Amended) A pair of molecular beacon probes comprising:

a first molecular beacon probe which is an oligonucleotide with a stem-loop structure having a first photoluminescent dye at one of the 5' or 3' ends and a quenching agent at the opposite 5' or 3' end, wherein the loop consists of 16 bases having a  $T_m$  of 50-51°C and the stem consists of 4 base pairs having a sequence 5'-CACG-3'; and

a second molecular beacon probe which is an oligonucleotide with a stem-loop structure having a second photoluminescent dye at one of the 5' or 3' ends and a quenching agent at the opposite 5' or 3' end, wherein the loop consists of 19-20 bases having a  $T_m$  of 54-56°C and the stem consists of 4 base pairs having a sequence 5'-CACG-3';



wherein the first and the second photoluminescent dyes are distinct.

65. (Amended) A molecular beacon probe comprising:

an oligonucleotide comprising a stem and a loop structure and having a photoluminescent dye at one of the 5' or 3' ends and a quenching agent at the opposite 5' or 3' end, wherein the loop consists of 14-26 bases and has a T<sub>m</sub> of 50-51°C, and wherein the stem consists of 4 base pairs having a sequence 5'-CACG-3'.

68. (Amended) A molecular beacon probe comprising:

an oligonucleotide comprising a stem and a loop structure and having a photoluminescent dye at one of the 5' or 3' ends and a quenching agent at the opposite 5' or 3' end, wherein the loop consists of 14-26 bases and has a T<sub>m</sub> of 54-56°C, and wherein the stem consists of 4-6 base pairs comprising a sequence 5'-CACG-3'.

69. (Amended) A pair of molecular beacon probes comprising:

a first oligonucleotide comprising a first stem and a first loop structure and having a photoluminescent dye at one of the 5' or 3' ends and a quenching agent at the opposite 5' or 3' end, wherein the first loop consists of 14-26 bases and has a T<sub>m</sub> of 50-51°C, and wherein the first stem consists of 4 base pairs having a sequence 5'-CACG-3'; and

a second oligonucleotide comprising a second stem and a second loop structure and having a photoluminescent dye at one of the 5' or 3' ends and a quenching agent at the opposite 5' or 3' end, wherein the second loop consists of 14-26 bases and has a T<sub>m</sub>



of 54-56°C, and wherein the second stem consists of 4-6 base pairs comprising a sequence 5'-CACG-3'.

#### REMARKS

##### The Invention

The invention is directed to methods for determining the ratio of a selected genetic sequence in a population of genetic sequences.

The invention is also drawn to molecular beacon probes. The molecular beacon probes can be used to execute the methods of the invention. The molecular beacon probes comprise an oligonucleotide comprising a stem and a loop structure and have a photoluminescent dye at one of the 5' or 3' ends and a quenching agent at the opposite 5' or 3' end.

##### The Amendments

Claims 33, 36-37, 65, and 68-69 have each been amended to recite that the loops of the molecular beacon probes consist of a specified number of "bases" instead of "base pairs." The amendments are supported by the specification and drawings of the application as filed. The specification supports this amendment where it discloses the sequence of two example molecular beacon probes: "MB-RED: 5'-Cy3'-CACGGCCTGCTGAAAATGACTGCGTG-Dabcyl-3'; MB-GREEN: 5'-Fluorescein-CACGGGAGCTGGTGGCGTAGCGTG-Dabcyl-3'." (Page 15, lines 1-3.) Each of the molecular beacon probes has a 5' terminal sequence, 5'-CACG-3', which base pairs with

the 3' terminal sequence, 5'-CGTG-3', to form the stem of the probe. The intervening sequence of each probe forms the loop. The loop of each probe is not self-complementary and therefore does not form base pairs. Thus the loop is measured in bases rather than base pairs. Figure 1b is a further disclosure that the loop of the molecular beacon probes is not base paired. Figure 1b is an illustration of the stem-loop structure of a molecular beacon probe. The stem portion of the structure, or bottom half, is base paired. The loop, above the stem and at the top half of the probe, is not base paired. Therefore the drawings also support that the loop should be measured in bases, not base pairs. Thus the amendment to the claims is supported by the application. The amendments do not introduce new matter and do not require a new search. The amendments also clarify the claims and do not narrow the scope of the claims. The amendments were not earlier introduced, as applicants were just became of this inadvertent mistake.

Claim 65 has been amended to recite that the loop of the molecular beacon probe "consists of 14-26 bases" instead of "comprises 16 base pairs." Claim 65 has also been amended to recite that the stem of a molecular beacon probe "consists of 4 base pairs" instead of "comprises 4 base pairs." These amendments are supported by the specification where it is disclosed, "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." (Page 18, lines 6-8.) Thus, molecular beacon probes with loops consisting of 14-26 bases and stems consisting of 4 base pairs are supported in the specification. The amendments therefore introduce no new matter and

do not require a new search. The amendments to claim 65 were not made earlier as it is a newly entered claim and applicants have first been made aware of its alleged insufficiencies in the final rejection. The amendments are also believed to place the claims in condition for allowance or better condition for appeal.

Claim 68 has been similarly amended to recite the loop of the molecular beacon probe "consists of 14-26 bases" instead of "comprises 19-20 base pairs." Claim 68 has also been amended to recite the stem of the molecular beacon probe "consists of 4-6 bases" instead of "comprises 4 base pairs." These amendments are also supported by the specification where it is disclosed, "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." (Page 18, lines 6-8.) Thus, molecular beacon probes with loops consisting of 14-26 bases and stems consisting of 4 to 6 base pairs are supported in the specification. The amendments therefore introduce no new matter and do not require a new search. The amendments to claim 68 were not made earlier as it is a newly entered claim and applicants have first been made aware of its alleged insufficiencies in the final rejection. The amendments also are believed to place the claims in condition for allowance or better condition for appeal.

Claim 69 has been similarly amended to recite that the first oligonucleotide of a pair of molecular beacon probes has a first loop that "consists of 14-26 bases" instead of "comprises 16 base pairs" and a first stem that "consists of 4 base pairs" instead of "comprises 4 base pairs." Claim 69 has also been amended to recite that the second oligonucleotide of the pair of molecular beacon probes has a second loop that "consists of

14-26 bases" instead of "comprises 19-20 base pairs" and a stem that "consists of 4-6 base pairs" instead of "comprises 4 base pairs." This amendment is also supported by the specification at page 18, lines 6-8. Thus, molecular beacon probes with loops consisting of 14-26 bases and stems consisting of 4 to 6 base pairs are supported in the specification. The amendments to claim 68 therefore introduce no new matter and do not require a new search. These amendments were not made earlier as it is a newly entered claim and applicants have first been made aware of its alleged insufficiencies in the final rejection. These amendments to claim 68 are also believed to place the claim in condition for allowance or in better condition for appeal.

The Rejection of Claims 65-69 under 35 U.S.C. § 112

Claims 65-69 have been rejected under 35 U.S.C. § 112, first paragraph, as containing subject matter which was not described in the specification in such a way as to reasonably convey to one skilled in the relevant art that the inventor(s), at the time the application was filed, had possession of the claimed invention. Specifically, the Office Action alleges that the "specification lacks support for molecular beacon that has a loop greater than 16 base pairs with  $T_m$  of 50-51°C and stem comprising 4 base pairs nor a molecular beacon that a loop comprising 19-20 base pairs and  $T_m$  of 54-56°C and stem comprising of 4 base pairs." (Paper 10, page 3, lines 5-7.) Applicants respectfully traverse.

To satisfy the written description requirement, a patent specification must describe the claimed invention in sufficient detail that one skilled in the art can

reasonably conclude that the inventor had possession of the claimed invention. *Vas-Cath, Inc. v. Mahurkar*, 935 F.2d. at 1563. Amended claims 65-69 are described in the specification such that one of skill in the art would conclude that the inventors had possession of the invention.

Claims 65, 68, and 69 have been amended to recite molecular beacon probes that have a loop that "consists of 14-26 bases." Claims 65, 68, and 69 have each also been amended to recite that the molecular beacon probes have a stem that "consists of 4 base pairs" or "consists of 4-6 base pairs." The inventors clearly had possession of the invention as recited in the amended claims. The specification discloses that "[l]oops 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." (Page 18, lines 6-8.) Thus the specification discloses that molecular beacon probes with loops consisting of 14 to 26 bases and stems consisting of 4-6 bases are of the lengths that are optimum in probe design. Clearly the inventors had possession of the invention as it is claimed. Withdrawal of this rejection to claims 65, 68, 69 and dependent claims 66-67 is respectfully requested.

The Rejection of Claim 68 under 35 U.S.C. § 112

Claims 65-69 have been 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. Specifically, the Office Action asserts that the phrase "comprising 19-20" renders claim 68 unclear. Claim 68 has been amended to

recite "consists of 14-26" in place of "comprises 19-20." Thus the rejection is rendered moot.

Rejection of Claims 65-67 under 35 U.S.C. §102(e)

Claims 65-67 are rejected under 35 U.S.C. §102(e) as being anticipated by Tyagi et al. (U.S. 5,925,517 March 14, 2000).

The Office Action asserts that Tyagi et al. teaches a molecular beacon probe with a stem comprising CACG (see col. 11 probe 3) and a loop of Tm 50 (see SEQ ID NO: 3 at column 12). (Paper 10, page 4, lines 8-9.) Applicants respectfully traverse.

Applicants are unable to locate the cited molecular beacon probe in Tyagi et al (U.S. 5,925,517). However, Tyagi et al., U.S. 6,037,130, issued March 14, 2000, does teach a molecular beacon probe (probe 3) at column 11. Applicants will discuss molecular beacon probe 3 disclosed in Tyagi et al., U.S. 5,925,517 in this response.

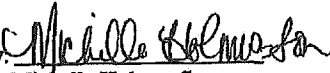
To reject a claim under 35 U.S.C. § 102, each element must be taught or inherently described in the prior art reference. "A claim is anticipated only if each and every element as set forth in the claim is found, either expressly or inherently described, in a single prior art reference." *Verdegaal Bros. v. Union oil Co. of California*, 814 F.2d 628, (Fed. Cir. 1987). Tyagi et al. do not teach each element as set forth in claims 65-67.

Claim 65 has been amended to recite a molecular beacon probe "wherein the loop consists of 14-26 bases and has a Tm of 50-51°, and wherein the stem consists of 4 base pairs having a sequence 5'-CACG-3.'" (Emphasis added.) Tyagi et al. do not teach a molecular beacon probe with the limitation of a stem consisting of 4 base pairs. Tyagi et

al. teach a molecular beacon probe of SEQ ID NO: 3 that has the sequence "TMR-5'-CCACGT-fluorescein-TCTTGTGGGTCAACCCCGTGG-3'-DABSYL." (Column 11 through column 12, line 40, emphasis in reference.) Thus Tyagi et al. teach a molecular beacon probe with a stem loop of 5 base pairs comprising the sequence CACG. Thus Tyagi et al. do not teach all the limitations of claim 65. Tyagi et al. do not teach a molecular beacon probe with a stem consisting of 4 base pairs. Withdrawal of this rejection to claims 65 and dependent claims 66-67 is respectfully requested.

Respectfully submitted,

Date: December 6, 2001

By:   
Michelle Holmes-Son  
Registration No. 47,660

Banner & Witcoff, Ltd.  
1001 G Street, NW  
Washington, DC 20001  
202-508-9100

**MARKED UP VERSION OF THE CLAIMS TO SHOW CHANGES MADE**

33. (Amended) A molecular beacon probe comprising:

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, wherein the loop consists of 16 bases [pairs], wherein the loop has a  $T_m$  of 50-51°C and the stem consists of 4 base pairs having a sequence 5'-CACG-3'.

36. (Amended) A molecular beacon probe comprising:

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, wherein the loop consists of 19-20 bases [pairs], wherein the loop has a  $T_m$  of 54-56°C and the stem consists of 4 base pairs having a sequence 5'-CACG-3'.

37. (Amended) A pair of molecular beacon probes comprising:

a first molecular beacon probe which is an oligonucleotide with a stem-loop structure having a first photoluminescent dye at one of the 5' or 3' ends and a quenching agent at the opposite 5' or 3' end, wherein the loop consists of 16 bases [pairs] having a  $T_m$  of 50-51°C and the stem consists of 4 base pairs having a sequence 5'-CACG-3'; and

a second molecular beacon probe which is an oligonucleotide with a stem-loop structure having a second photoluminescent dye at one of the 5' or 3' ends and a quenching agent at the opposite 5' or 3' end, wherein the loop consists of 19-20 bases



[pairs] having a  $T_m$  of 54-56°C and the stem consists of 4 base pairs having a sequence 5'-CACG-3';

wherein the first and the second photoluminescent dyes are distinct.

65. (Amended) A molecular beacon probe comprising:

an oligonucleotide comprising a stem and a loop structure and having a photoluminescent dye at one of the 5' or 3' ends and a quenching agent at the opposite 5' or 3' end, wherein the loop [comprises 16] consists of 14-26 bases [pairs] and has a  $T_m$  of 50-51°C, and wherein the stem [comprises] consists of 4 base pairs having a sequence 5'-CACG-3'.

68. (Amended) A molecular beacon probe comprising:

an oligonucleotide comprising a stem and a loop structure and having a photoluminescent dye at one of the 5' or 3' ends and a quenching agent at the opposite 5' or 3' end, wherein the loop [comprises 19-20] consists of 14-26 bases [pairs] and has a  $T_m$  of 54-56°C, and wherein the stem [comprises] consists of 4-6 base pairs [having] comprising a sequence 5'-CACG-3'.

69. (Amended) A pair of molecular beacon probes comprising:

a first oligonucleotide comprising a first stem and a first loop structure and having a photoluminescent dye at one of the 5' or 3' ends and a quenching agent at the opposite 5' or 3' end, wherein the first loop [comprises 16] consists of 14-26 bases [pairs] and has

a T<sub>m</sub> of 50-51°C, and wherein the first stem [comprises] consists of 4 base pairs having a sequence 5'-CACG-3'; and

a second oligonucleotide comprising a second stem and a second loop structure and having a photoluminescent dye at one of the 5' or 3' ends and a quenching agent at the opposite 5' or 3' end, wherein the second loop [comprises 19-20] consists of 14-26 bases [pairs] and has a T<sub>m</sub> of 54-56°C, and wherein the second stem [comprises] consists of 4-6 base pairs [having] comprising a sequence 5'-CACG-3'.

AF/1656

Please type a plus sign (+) inside this box →

Approved for use through 10/31/2002. OMB 0851-0031  
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PTO/SB/21 (08-00)



### TRANSMITTAL FORM

is used for all correspondence after initial filing)

|                        |                      |
|------------------------|----------------------|
| Application Number     | 09/613,826           |
| Filing Date            | July 11, 2000        |
| First Named Inventor   | Bert Vogelstein, et. |
| Group Art Unit         | 1856                 |
| Examiner Name          | J. Stew              |
| Attorney Docket Number | 01107.00031          |

Total Number of Pages in This Submission 13

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#### ENCLOSURES (check all that apply)

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| <input type="checkbox"/> Fee Attached                                        | <input type="checkbox"/> Drawing(s)                                                     | <input type="checkbox"/> Appeal Communication to Board of Appeals and Interferences        |
| <input checked="" type="checkbox"/> Amendment / Response                     | <input type="checkbox"/> Licensing-related Papers                                       | <input type="checkbox"/> Appeal Communication to Group (Appeal Notice, Brief, Reply Brief) |
| <input checked="" type="checkbox"/> After Final                              | <input type="checkbox"/> Petition Routing Slip (PTO/SB/69) and Accompanying Petition    | <input type="checkbox"/> Proprietary Information                                           |
| <input type="checkbox"/> Affidavits/declaration(s)                           | <input type="checkbox"/> Petition to Convert to a Provisional Application               | <input type="checkbox"/> Status Letter                                                     |
| <input type="checkbox"/> Extension of Time Request                           | <input type="checkbox"/> Power of Attorney, Revocation Change of Correspondence Address | <input type="checkbox"/> Other Enclosure(s) (please identify below):                       |
| <input type="checkbox"/> Express Abandonment Request                         | <input type="checkbox"/> Terminal Disclaimer                                            |                                                                                            |
| <input type="checkbox"/> Information Disclosure Statement                    | <input type="checkbox"/> Request for Refund                                             |                                                                                            |
| <input type="checkbox"/> Certified Copy of Priority Document(s)              | <input type="checkbox"/> CD, Number of CD(s) _____                                      |                                                                                            |
| <input type="checkbox"/> Response to Missing Parts/Incomplete Application    | Remarks                                                                                 |                                                                                            |
| <input type="checkbox"/> Response to Missing Parts under 37 CFR 1.52 or 1.53 |                                                                                         |                                                                                            |

#### SIGNATURE OF APPLICANT, ATTORNEY, OR AGENT

|                         |                                              |
|-------------------------|----------------------------------------------|
| Firm or Individual name | Michelle Holmes-Son, Registration No. 47,680 |
| Signature               | <i>Michelle Holmes-Son</i>                   |
| Date                    | December 6, 2001                             |

#### CERTIFICATE OF MAILING

I hereby certify that this correspondence is being deposited with the United States Postal Service as first class mail in an envelope addressed to: Assistant Commissioner for Patents, Washington, D.C. 20231 on this date: \_\_\_\_\_

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| Signature             |  |
| Date                  |  |

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| APPLICATION NO | FILING DATE | FIRST NAMED INVENTOR | ATTORNEY DOCKET NO | CONFIRMATION NO. |
|----------------|-------------|----------------------|--------------------|------------------|
| 09/613,826     | 07/11/2000  | Bert Vogelstein      | 01107.00031        | 9893             |

22907                      7590                      12/12/2001

**BANNER & WITCOFF**  
1001 G STREET N W  
SUITE 1100  
WASHINGTON, DC 20001

EXAMINER

SIEW, JEFFREY

ART UNIT                      PAPER NUMBER

1656

14

DATE MAILED: 12/13/2001

Please find below and/or attached an Office communication concerning this application or proceeding.

copy

|                        |                               |                                   |  |
|------------------------|-------------------------------|-----------------------------------|--|
| <b>Advisory Action</b> | Application No.<br>09/613,826 | Applicant(s)<br>VOGELSTEIN ET AL. |  |
|                        | Examiner<br>Jeffrey Siew      | Art Unit<br>1656                  |  |

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

THE REPLY FILED 06 December 2001 FAILS TO PLACE THIS APPLICATION IN CONDITION FOR ALLOWANCE. Therefore, further action by the applicant is required to avoid abandonment of this application. A proper reply to a final rejection under 37 CFR 1.113 may only be either: (1) a timely filed amendment which places the application in condition for allowance; (2) a timely filed Notice of Appeal (with appeal fee); or (3) a timely filed Request for Continued Examination (RCE) in compliance with 37 CFR 1.114.

**PERIOD FOR REPLY (check either a) or b))**

- 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.  
ONLY CHECK THIS BOX WHEN THE FIRST REPLY WAS FILED WITHIN TWO MONTHS OF THE FINAL REJECTION. 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) 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).

- 1.  A Notice of Appeal was filed on \_\_\_\_\_. Appellant's Brief must be filed within the period set forth in 37 CFR 1.192(a), or any extension thereof (37 CFR 1.191(d)), to avoid dismissal of the appeal.
- 2.  The proposed amendment(s) 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: the limitation to consisting of 14-26 would require new search and consideration.

- 3.  Applicant's reply has overcome the following rejection(s): See Continuation Sheet.
- 4.  Newly proposed or amended claim(s) \_\_\_\_\_ would be allowable if submitted in a separate, timely filed amendment canceling the non-allowable claim(s).
- 5.  The a)  affidavit, b)  exhibit, or c)  request for reconsideration has been considered but does NOT place the application in condition for allowance because: \_\_\_\_\_.
- 6.  The affidavit or exhibit will NOT be considered because it is not directed SOLELY to issues which were newly raised by the Examiner in the final rejection.
- 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.

The status of the claim(s) is (or will be) as follows:

Claim(s) allowed: 1-64.  
 Claim(s) objected to: \_\_\_\_\_  
 Claim(s) rejected: 65-69.  
 Claim(s) withdrawn from consideration: \_\_\_\_\_.

- 8.  The proposed drawing correction filed on \_\_\_\_\_ is a)  approved or b)  disapproved by the Examiner.
- 9.  Note the attached Information Disclosure Statement(s) (PTO-1449) Paper No(s). \_\_\_\_\_.
- 10.  Other: \_\_\_\_\_

Continuation Sheet (PTO-303)

Application No. 09/613,826

Continuation of 3. Applicant's reply has overcome the following rejection(s): the proposed amendment would overcome the 112 first written description and second paragraph rejections. The office would like to thank applicant to bringing attention the inadvertent oversight of 102(e) rejection over Tyagi US6,037,130. The 102(e) rejection appears overcome by the new amendment.

*Jeffrey Law*  
*3/23/02*  
*copy filed 12/13/01*



#15  
2/B  
1/31/02

PATENT APPLICATION

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

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|                            |   |                                 |
|----------------------------|---|---------------------------------|
| In Re Application of:      | ) |                                 |
|                            | ) |                                 |
| Bert VOGELSTEIN et al.     | ) |                                 |
|                            | ) | Group Art Unit: 1656            |
| Serial No. 09/613,826      | ) |                                 |
|                            | ) | Examiner: J. Siew               |
| Filed: July 11, 2000       | ) | Box: AF                         |
|                            | ) |                                 |
| For: DIGITAL AMPLIFICATION | ) | Attorney Docket No. 01107.00031 |

INFORMATION DISCLOSURE STATEMENT

Assistant Commissioner for Patents  
Washington, D.C. 20231

Sir:

Pursuant to 37 C.F.R. § 1.56 and in compliance with 37 C.F.R. § 1.97(d), Applicants herewith submit Form PTO 1449 listing references for consideration in connection with the above-identified application. A copy of each reference is provided herewith.

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.


01/28/2002 1600/2900 00000149 190733 09613026  
01 FC:126 100.00 CH

Serial No. 09/613,826

Applicants respectfully request that the Examiner consider and enter these documents into the file of the above-identified application. A fee of \$180.00 as set forth in 37 C.F.R. § 1.17(p) is enclosed herewith to ensure consideration and entry of the cited documents by the Examiner. If any additional fee is deemed necessary, the Commissioner is authorized to charge our Deposit Account No. 19-0733.

Respectfully submitted,

Date: January 23, 2002

By:   
Michelle L. Holmes-Son  
Registration No. 47,660

BANNER & WITCOFF, LTD  
1001 G Street, N.W.  
Eleventh Floor  
Washington, D.C. 20001-4597  
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|                                                                                                                                                          |  |                                                                                                                                                                                                                                                                            |  |
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| Substitute for form 1449A/PTO<br><b>INFORMATION DISCLOSURE STATEMENT BY APPLICANT</b><br>(use as many sheets as necessary)<br>Sheet <u>1</u> of <u>2</u> |  | <b>Complete if Known</b><br>Application Number <u>09/613,826</u><br>Filing Date <u>July 11, 2000</u><br>First Named Inventor <u>Bert Vogelstein et al.</u><br>Group Art Unit <u>1800-1697</u><br>Examiner Name <u>J. Siew</u><br>Attorney Docket Number <u>01107.00031</u> |  |
|----------------------------------------------------------------------------------------------------------------------------------------------------------|--|----------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------|--|

INFO  
 JAN 23 2002  
 TRADEMARK OFFICE

| OTHER PRIOR ART -- NON PATENT LITERATURE DOCUMENTS |                       |                                                                                                                                                                                                                                                                 |                |
|----------------------------------------------------|-----------------------|-----------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------|----------------|
| Examiner Initials *                                | Cite No. <sup>1</sup> | Include name of the author (in CAPITAL LETTERS), title of the article (when appropriate), title of the item (book, magazine, journal, serial, symposium, catalog, etc.), date, page(s), volume-issue number(s), publisher, city and/or country where published. | T <sup>2</sup> |
| JS                                                 |                       | A. PIATEK et al., "Molecular Beacon Sequence Analysis for Detecting Drug Resistance in Mycobacterium Tuberculosis", Nature Biotechnology, April 1999, pp. 359-363, Vol. 18, No. 4                                                                               |                |
|                                                    |                       | S. TYAGI et al., "Multicolor Molecular Beacons for allele discrimination", Nature Biotechnology, pp. 303-308, January 1998, Vol. 16, No. 1                                                                                                                      |                |
|                                                    |                       | J. A.M. VET et al., "Multitox Detection of Four Pathogenic Retroviruses Using Molecular Beacons", Proceedings of the National Academy of Sciences of the United States, May 25, 1999, pp. 6394-6399, Vol. 96, No. 11                                            |                |
|                                                    |                       | S. TYAGI et al., "Molecular Beacons: probes that Fluoresce Upon Hybridization", Nature Biotechnology, 1996, pp. 303-308, Vol. 14, No. 3                                                                                                                         |                |
|                                                    |                       | W. P. HALFORD et al., "The Inherent Quantitative Capacity of the Reverse Transcription-Polymerase Chain Reaction", Analytical Biochemistry, January 15, 1999, pp. 181-191, Vol. 266, No. 2                                                                      |                |
| JS                                                 |                       | B. VOGELSTEIN et al., "Digital PCR", Proceedings of the National Academy of Sciences of the United States, August 3, 1999, pp. 9238-9241, Vol. 96, No. 16                                                                                                       |                |
|                                                    |                       | K. D.E. EVERETT et al., "Identification of nine species of the Chlamydiaceae Using PCR-RFLP", April 1999, pp. 803-813, Vol. 49, No. 2                                                                                                                           |                |

|                    |                |                 |                |
|--------------------|----------------|-----------------|----------------|
| Examiner Signature | <i>J. Siew</i> | Date Considered | <u>3/18/02</u> |
|--------------------|----------------|-----------------|----------------|

<sup>1</sup> EXAMINER: Initial if reference considered, whether or not citation is in conformance with MPEP 809. Draw line through citation if not in conformance and not considered. Include copy of this form with next communication to applicant.  
<sup>2</sup> Unique citation designation number (optional). <sup>3</sup> Applicant is to place a check mark here if English language Translation is attached.  
 Burden Hour Statement: This form is estimated to take 2.0 hours to complete. Time will vary depending upon the needs of the individual case. Any comments on the amount of time you are required to complete this form should be sent to the Chief Information Officer, U.S. Patent and Trademark Office, Washington, DC 20231. DO NOT SEND FEES OR COMPLETED FORMS TO THIS ADDRESS. SEND TO: Assistant Commissioner for Patents, Washington, DC 20231.

## FEE TRANSMITTAL for FY 2002

*Patent fees are subject to annual revision.*

*Complete if Known*

|                     |                        |
|---------------------|------------------------|
| Application Number  | 09/813,826             |
| Filing Date         | July 11, 2000          |
| Ret Named Inventor  | Bert Vogelstein et al. |
| Examiner Name       | J. Siew                |
| Group / Art Unit    | 1658                   |
| Attorney Docket No. | 01107.00031            |

**TOTAL AMOUNT OF PAYMENT (\$)** 180

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| METHOD OF PAYMENT (check all that apply)                                                                                                                                                                                                                                                                                                                                                                                                    |                            | FEE CALCULATION (continued)                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                               |                                                            |                            |                            |                       |                 |          |           |     |                        |    |     |           |     |                                   |    |     |           |     |                                       |     |     |           |       |                                                    |       |     |           |      |                                                            |      |  |     |        |     |        |  |     |     |     |    |  |     |     |     |     |  |     |     |     |     |  |     |       |     |     |  |     |       |     |     |  |     |     |     |     |  |     |     |     |     |  |     |     |     |     |  |     |       |     |       |  |     |     |     |    |  |     |       |     |     |  |     |       |     |     |  |     |     |     |     |  |     |     |     |     |  |     |     |     |     |  |     |    |     |    |  |     |     |     |     |     |     |    |     |    |  |     |     |     |     |  |     |     |     |     |  |     |     |     |     |  |     |     |     |     |  |
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| <input type="checkbox"/> Check <input type="checkbox"/> Credit card <input type="checkbox"/> Money Order <input type="checkbox"/> Other <input type="checkbox"/> None                                                                                                                                                                                                                                                                       |                            | <b>3. ADDITIONAL FEES</b>                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                 |                                                            |                            |                            |                       |                 |          |           |     |                        |    |     |           |     |                                   |    |     |           |     |                                       |     |     |           |       |                                                    |       |     |           |      |                                                            |      |  |     |        |     |        |  |     |     |     |    |  |     |     |     |     |  |     |     |     |     |  |     |       |     |     |  |     |       |     |     |  |     |     |     |     |  |     |     |     |     |  |     |     |     |     |  |     |       |     |       |  |     |     |     |    |  |     |       |     |     |  |     |       |     |     |  |     |     |     |     |  |     |     |     |     |  |     |     |     |     |  |     |    |     |    |  |     |     |     |     |     |     |    |     |    |  |     |     |     |     |  |     |     |     |     |  |     |     |     |     |  |     |     |     |     |  |
| <input checked="" type="checkbox"/> Deposit Account:                                                                                                                                                                                                                                                                                                                                                                                        |                            | <table border="1" style="width: 100%; border-collapse: collapse;"> <thead> <tr> <th>Fee Code</th> <th>Large Entity Fee (\$)</th> <th>Small Entity Fee (\$)</th> <th>Fee Description</th> <th>Fee Paid</th> </tr> </thead> <tbody> <tr><td>105</td><td>130</td><td>205</td><td>65</td><td></td></tr> <tr><td>127</td><td>80</td><td>227</td><td>25</td><td></td></tr> <tr><td>139</td><td>130</td><td>130</td><td>130</td><td></td></tr> <tr><td>147</td><td>2,620</td><td>147</td><td>2,620</td><td></td></tr> <tr><td>112</td><td>920*</td><td>112</td><td>920*</td><td></td></tr> <tr><td>113</td><td>1,840*</td><td>113</td><td>1,840*</td><td></td></tr> <tr><td>115</td><td>110</td><td>215</td><td>55</td><td></td></tr> <tr><td>116</td><td>400</td><td>210</td><td>200</td><td></td></tr> <tr><td>117</td><td>920</td><td>217</td><td>480</td><td></td></tr> <tr><td>118</td><td>1,440</td><td>218</td><td>720</td><td></td></tr> <tr><td>128</td><td>1,960</td><td>228</td><td>980</td><td></td></tr> <tr><td>119</td><td>320</td><td>219</td><td>160</td><td></td></tr> <tr><td>120</td><td>320</td><td>220</td><td>160</td><td></td></tr> <tr><td>121</td><td>280</td><td>221</td><td>140</td><td></td></tr> <tr><td>138</td><td>1,510</td><td>138</td><td>1,510</td><td></td></tr> <tr><td>140</td><td>110</td><td>240</td><td>55</td><td></td></tr> <tr><td>141</td><td>1,280</td><td>241</td><td>640</td><td></td></tr> <tr><td>142</td><td>1,280</td><td>242</td><td>640</td><td></td></tr> <tr><td>143</td><td>480</td><td>243</td><td>230</td><td></td></tr> <tr><td>144</td><td>620</td><td>244</td><td>310</td><td></td></tr> <tr><td>122</td><td>130</td><td>122</td><td>130</td><td></td></tr> <tr><td>123</td><td>80</td><td>123</td><td>50</td><td></td></tr> <tr><td>128</td><td>180</td><td>128</td><td>180</td><td>180</td></tr> <tr><td>581</td><td>40</td><td>581</td><td>40</td><td></td></tr> <tr><td>148</td><td>740</td><td>248</td><td>370</td><td></td></tr> <tr><td>149</td><td>740</td><td>249</td><td>370</td><td></td></tr> <tr><td>178</td><td>740</td><td>270</td><td>370</td><td></td></tr> <tr><td>189</td><td>900</td><td>189</td><td>900</td><td></td></tr> </tbody> </table> |                                                            | Fee Code                   | Large Entity Fee (\$)      | Small Entity Fee (\$) | Fee Description | Fee Paid | 105       | 130 | 205                    | 65 |     | 127       | 80  | 227                               | 25 |     | 139       | 130 | 130                                   | 130 |     | 147       | 2,620 | 147                                                | 2,620 |     | 112       | 920* | 112                                                        | 920* |  | 113 | 1,840* | 113 | 1,840* |  | 115 | 110 | 215 | 55 |  | 116 | 400 | 210 | 200 |  | 117 | 920 | 217 | 480 |  | 118 | 1,440 | 218 | 720 |  | 128 | 1,960 | 228 | 980 |  | 119 | 320 | 219 | 160 |  | 120 | 320 | 220 | 160 |  | 121 | 280 | 221 | 140 |  | 138 | 1,510 | 138 | 1,510 |  | 140 | 110 | 240 | 55 |  | 141 | 1,280 | 241 | 640 |  | 142 | 1,280 | 242 | 640 |  | 143 | 480 | 243 | 230 |  | 144 | 620 | 244 | 310 |  | 122 | 130 | 122 | 130 |  | 123 | 80 | 123 | 50 |  | 128 | 180 | 128 | 180 | 180 | 581 | 40 | 581 | 40 |  | 148 | 740 | 248 | 370 |  | 149 | 740 | 249 | 370 |  | 178 | 740 | 270 | 370 |  | 189 | 900 | 189 | 900 |  |
| Fee Code                                                                                                                                                                                                                                                                                                                                                                                                                                    | Large Entity Fee (\$)      | Small Entity Fee (\$)                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                     | Fee Description                                            | Fee Paid                   |                            |                       |                 |          |           |     |                        |    |     |           |     |                                   |    |     |           |     |                                       |     |     |           |       |                                                    |       |     |           |      |                                                            |      |  |     |        |     |        |  |     |     |     |    |  |     |     |     |     |  |     |     |     |     |  |     |       |     |     |  |     |       |     |     |  |     |     |     |     |  |     |     |     |     |  |     |     |     |     |  |     |       |     |       |  |     |     |     |    |  |     |       |     |     |  |     |       |     |     |  |     |     |     |     |  |     |     |     |     |  |     |     |     |     |  |     |    |     |    |  |     |     |     |     |     |     |    |     |    |  |     |     |     |     |  |     |     |     |     |  |     |     |     |     |  |     |     |     |     |  |
| 105                                                                                                                                                                                                                                                                                                                                                                                                                                         | 130                        | 205                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                       | 65                                                         |                            |                            |                       |                 |          |           |     |                        |    |     |           |     |                                   |    |     |           |     |                                       |     |     |           |       |                                                    |       |     |           |      |                                                            |      |  |     |        |     |        |  |     |     |     |    |  |     |     |     |     |  |     |     |     |     |  |     |       |     |     |  |     |       |     |     |  |     |     |     |     |  |     |     |     |     |  |     |     |     |     |  |     |       |     |       |  |     |     |     |    |  |     |       |     |     |  |     |       |     |     |  |     |     |     |     |  |     |     |     |     |  |     |     |     |     |  |     |    |     |    |  |     |     |     |     |     |     |    |     |    |  |     |     |     |     |  |     |     |     |     |  |     |     |     |     |  |     |     |     |     |  |
| 127                                                                                                                                                                                                                                                                                                                                                                                                                                         | 80                         | 227                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                       | 25                                                         |                            |                            |                       |                 |          |           |     |                        |    |     |           |     |                                   |    |     |           |     |                                       |     |     |           |       |                                                    |       |     |           |      |                                                            |      |  |     |        |     |        |  |     |     |     |    |  |     |     |     |     |  |     |     |     |     |  |     |       |     |     |  |     |       |     |     |  |     |     |     |     |  |     |     |     |     |  |     |     |     |     |  |     |       |     |       |  |     |     |     |    |  |     |       |     |     |  |     |       |     |     |  |     |     |     |     |  |     |     |     |     |  |     |     |     |     |  |     |    |     |    |  |     |     |     |     |     |     |    |     |    |  |     |     |     |     |  |     |     |     |     |  |     |     |     |     |  |     |     |     |     |  |
| 139                                                                                                                                                                                                                                                                                                                                                                                                                                         | 130                        | 130                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                       | 130                                                        |                            |                            |                       |                 |          |           |     |                        |    |     |           |     |                                   |    |     |           |     |                                       |     |     |           |       |                                                    |       |     |           |      |                                                            |      |  |     |        |     |        |  |     |     |     |    |  |     |     |     |     |  |     |     |     |     |  |     |       |     |     |  |     |       |     |     |  |     |     |     |     |  |     |     |     |     |  |     |     |     |     |  |     |       |     |       |  |     |     |     |    |  |     |       |     |     |  |     |       |     |     |  |     |     |     |     |  |     |     |     |     |  |     |     |     |     |  |     |    |     |    |  |     |     |     |     |     |     |    |     |    |  |     |     |     |     |  |     |     |     |     |  |     |     |     |     |  |     |     |     |     |  |
| 147                                                                                                                                                                                                                                                                                                                                                                                                                                         | 2,620                      | 147                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                       | 2,620                                                      |                            |                            |                       |                 |          |           |     |                        |    |     |           |     |                                   |    |     |           |     |                                       |     |     |           |       |                                                    |       |     |           |      |                                                            |      |  |     |        |     |        |  |     |     |     |    |  |     |     |     |     |  |     |     |     |     |  |     |       |     |     |  |     |       |     |     |  |     |     |     |     |  |     |     |     |     |  |     |     |     |     |  |     |       |     |       |  |     |     |     |    |  |     |       |     |     |  |     |       |     |     |  |     |     |     |     |  |     |     |     |     |  |     |     |     |     |  |     |    |     |    |  |     |     |     |     |     |     |    |     |    |  |     |     |     |     |  |     |     |     |     |  |     |     |     |     |  |     |     |     |     |  |
| 112                                                                                                                                                                                                                                                                                                                                                                                                                                         | 920*                       | 112                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                       | 920*                                                       |                            |                            |                       |                 |          |           |     |                        |    |     |           |     |                                   |    |     |           |     |                                       |     |     |           |       |                                                    |       |     |           |      |                                                            |      |  |     |        |     |        |  |     |     |     |    |  |     |     |     |     |  |     |     |     |     |  |     |       |     |     |  |     |       |     |     |  |     |     |     |     |  |     |     |     |     |  |     |     |     |     |  |     |       |     |       |  |     |     |     |    |  |     |       |     |     |  |     |       |     |     |  |     |     |     |     |  |     |     |     |     |  |     |     |     |     |  |     |    |     |    |  |     |     |     |     |     |     |    |     |    |  |     |     |     |     |  |     |     |     |     |  |     |     |     |     |  |     |     |     |     |  |
| 113                                                                                                                                                                                                                                                                                                                                                                                                                                         | 1,840*                     | 113                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                       | 1,840*                                                     |                            |                            |                       |                 |          |           |     |                        |    |     |           |     |                                   |    |     |           |     |                                       |     |     |           |       |                                                    |       |     |           |      |                                                            |      |  |     |        |     |        |  |     |     |     |    |  |     |     |     |     |  |     |     |     |     |  |     |       |     |     |  |     |       |     |     |  |     |     |     |     |  |     |     |     |     |  |     |     |     |     |  |     |       |     |       |  |     |     |     |    |  |     |       |     |     |  |     |       |     |     |  |     |     |     |     |  |     |     |     |     |  |     |     |     |     |  |     |    |     |    |  |     |     |     |     |     |     |    |     |    |  |     |     |     |     |  |     |     |     |     |  |     |     |     |     |  |     |     |     |     |  |
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| 116                                                                                                                                                                                                                                                                                                                                                                                                                                         | 400                        | 210                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                       | 200                                                        |                            |                            |                       |                 |          |           |     |                        |    |     |           |     |                                   |    |     |           |     |                                       |     |     |           |       |                                                    |       |     |           |      |                                                            |      |  |     |        |     |        |  |     |     |     |    |  |     |     |     |     |  |     |     |     |     |  |     |       |     |     |  |     |       |     |     |  |     |     |     |     |  |     |     |     |     |  |     |     |     |     |  |     |       |     |       |  |     |     |     |    |  |     |       |     |     |  |     |       |     |     |  |     |     |     |     |  |     |     |     |     |  |     |     |     |     |  |     |    |     |    |  |     |     |     |     |     |     |    |     |    |  |     |     |     |     |  |     |     |     |     |  |     |     |     |     |  |     |     |     |     |  |
| 117                                                                                                                                                                                                                                                                                                                                                                                                                                         | 920                        | 217                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                       | 480                                                        |                            |                            |                       |                 |          |           |     |                        |    |     |           |     |                                   |    |     |           |     |                                       |     |     |           |       |                                                    |       |     |           |      |                                                            |      |  |     |        |     |        |  |     |     |     |    |  |     |     |     |     |  |     |     |     |     |  |     |       |     |     |  |     |       |     |     |  |     |     |     |     |  |     |     |     |     |  |     |     |     |     |  |     |       |     |       |  |     |     |     |    |  |     |       |     |     |  |     |       |     |     |  |     |     |     |     |  |     |     |     |     |  |     |     |     |     |  |     |    |     |    |  |     |     |     |     |     |     |    |     |    |  |     |     |     |     |  |     |     |     |     |  |     |     |     |     |  |     |     |     |     |  |
| 118                                                                                                                                                                                                                                                                                                                                                                                                                                         | 1,440                      | 218                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                       | 720                                                        |                            |                            |                       |                 |          |           |     |                        |    |     |           |     |                                   |    |     |           |     |                                       |     |     |           |       |                                                    |       |     |           |      |                                                            |      |  |     |        |     |        |  |     |     |     |    |  |     |     |     |     |  |     |     |     |     |  |     |       |     |     |  |     |       |     |     |  |     |     |     |     |  |     |     |     |     |  |     |     |     |     |  |     |       |     |       |  |     |     |     |    |  |     |       |     |     |  |     |       |     |     |  |     |     |     |     |  |     |     |     |     |  |     |     |     |     |  |     |    |     |    |  |     |     |     |     |     |     |    |     |    |  |     |     |     |     |  |     |     |     |     |  |     |     |     |     |  |     |     |     |     |  |
| 128                                                                                                                                                                                                                                                                                                                                                                                                                                         | 1,960                      | 228                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                       | 980                                                        |                            |                            |                       |                 |          |           |     |                        |    |     |           |     |                                   |    |     |           |     |                                       |     |     |           |       |                                                    |       |     |           |      |                                                            |      |  |     |        |     |        |  |     |     |     |    |  |     |     |     |     |  |     |     |     |     |  |     |       |     |     |  |     |       |     |     |  |     |     |     |     |  |     |     |     |     |  |     |     |     |     |  |     |       |     |       |  |     |     |     |    |  |     |       |     |     |  |     |       |     |     |  |     |     |     |     |  |     |     |     |     |  |     |     |     |     |  |     |    |     |    |  |     |     |     |     |     |     |    |     |    |  |     |     |     |     |  |     |     |     |     |  |     |     |     |     |  |     |     |     |     |  |
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| 120                                                                                                                                                                                                                                                                                                                                                                                                                                         | 320                        | 220                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                       | 160                                                        |                            |                            |                       |                 |          |           |     |                        |    |     |           |     |                                   |    |     |           |     |                                       |     |     |           |       |                                                    |       |     |           |      |                                                            |      |  |     |        |     |        |  |     |     |     |    |  |     |     |     |     |  |     |     |     |     |  |     |       |     |     |  |     |       |     |     |  |     |     |     |     |  |     |     |     |     |  |     |     |     |     |  |     |       |     |       |  |     |     |     |    |  |     |       |     |     |  |     |       |     |     |  |     |     |     |     |  |     |     |     |     |  |     |     |     |     |  |     |    |     |    |  |     |     |     |     |     |     |    |     |    |  |     |     |     |     |  |     |     |     |     |  |     |     |     |     |  |     |     |     |     |  |
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| 138                                                                                                                                                                                                                                                                                                                                                                                                                                         | 1,510                      | 138                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                       | 1,510                                                      |                            |                            |                       |                 |          |           |     |                        |    |     |           |     |                                   |    |     |           |     |                                       |     |     |           |       |                                                    |       |     |           |      |                                                            |      |  |     |        |     |        |  |     |     |     |    |  |     |     |     |     |  |     |     |     |     |  |     |       |     |     |  |     |       |     |     |  |     |     |     |     |  |     |     |     |     |  |     |     |     |     |  |     |       |     |       |  |     |     |     |    |  |     |       |     |     |  |     |       |     |     |  |     |     |     |     |  |     |     |     |     |  |     |     |     |     |  |     |    |     |    |  |     |     |     |     |     |     |    |     |    |  |     |     |     |     |  |     |     |     |     |  |     |     |     |     |  |     |     |     |     |  |
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| 141                                                                                                                                                                                                                                                                                                                                                                                                                                         | 1,280                      | 241                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                       | 640                                                        |                            |                            |                       |                 |          |           |     |                        |    |     |           |     |                                   |    |     |           |     |                                       |     |     |           |       |                                                    |       |     |           |      |                                                            |      |  |     |        |     |        |  |     |     |     |    |  |     |     |     |     |  |     |     |     |     |  |     |       |     |     |  |     |       |     |     |  |     |     |     |     |  |     |     |     |     |  |     |     |     |     |  |     |       |     |       |  |     |     |     |    |  |     |       |     |     |  |     |       |     |     |  |     |     |     |     |  |     |     |     |     |  |     |     |     |     |  |     |    |     |    |  |     |     |     |     |     |     |    |     |    |  |     |     |     |     |  |     |     |     |     |  |     |     |     |     |  |     |     |     |     |  |
| 142                                                                                                                                                                                                                                                                                                                                                                                                                                         | 1,280                      | 242                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                       | 640                                                        |                            |                            |                       |                 |          |           |     |                        |    |     |           |     |                                   |    |     |           |     |                                       |     |     |           |       |                                                    |       |     |           |      |                                                            |      |  |     |        |     |        |  |     |     |     |    |  |     |     |     |     |  |     |     |     |     |  |     |       |     |     |  |     |       |     |     |  |     |     |     |     |  |     |     |     |     |  |     |     |     |     |  |     |       |     |       |  |     |     |     |    |  |     |       |     |     |  |     |       |     |     |  |     |     |     |     |  |     |     |     |     |  |     |     |     |     |  |     |    |     |    |  |     |     |     |     |     |     |    |     |    |  |     |     |     |     |  |     |     |     |     |  |     |     |     |     |  |     |     |     |     |  |
| 143                                                                                                                                                                                                                                                                                                                                                                                                                                         | 480                        | 243                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                       | 230                                                        |                            |                            |                       |                 |          |           |     |                        |    |     |           |     |                                   |    |     |           |     |                                       |     |     |           |       |                                                    |       |     |           |      |                                                            |      |  |     |        |     |        |  |     |     |     |    |  |     |     |     |     |  |     |     |     |     |  |     |       |     |     |  |     |       |     |     |  |     |     |     |     |  |     |     |     |     |  |     |     |     |     |  |     |       |     |       |  |     |     |     |    |  |     |       |     |     |  |     |       |     |     |  |     |     |     |     |  |     |     |     |     |  |     |     |     |     |  |     |    |     |    |  |     |     |     |     |     |     |    |     |    |  |     |     |     |     |  |     |     |     |     |  |     |     |     |     |  |     |     |     |     |  |
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| 122                                                                                                                                                                                                                                                                                                                                                                                                                                         | 130                        | 122                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                       | 130                                                        |                            |                            |                       |                 |          |           |     |                        |    |     |           |     |                                   |    |     |           |     |                                       |     |     |           |       |                                                    |       |     |           |      |                                                            |      |  |     |        |     |        |  |     |     |     |    |  |     |     |     |     |  |     |     |     |     |  |     |       |     |     |  |     |       |     |     |  |     |     |     |     |  |     |     |     |     |  |     |     |     |     |  |     |       |     |       |  |     |     |     |    |  |     |       |     |     |  |     |       |     |     |  |     |     |     |     |  |     |     |     |     |  |     |     |     |     |  |     |    |     |    |  |     |     |     |     |     |     |    |     |    |  |     |     |     |     |  |     |     |     |     |  |     |     |     |     |  |     |     |     |     |  |
| 123                                                                                                                                                                                                                                                                                                                                                                                                                                         | 80                         | 123                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                       | 50                                                         |                            |                            |                       |                 |          |           |     |                        |    |     |           |     |                                   |    |     |           |     |                                       |     |     |           |       |                                                    |       |     |           |      |                                                            |      |  |     |        |     |        |  |     |     |     |    |  |     |     |     |     |  |     |     |     |     |  |     |       |     |     |  |     |       |     |     |  |     |     |     |     |  |     |     |     |     |  |     |     |     |     |  |     |       |     |       |  |     |     |     |    |  |     |       |     |     |  |     |       |     |     |  |     |     |     |     |  |     |     |     |     |  |     |     |     |     |  |     |    |     |    |  |     |     |     |     |     |     |    |     |    |  |     |     |     |     |  |     |     |     |     |  |     |     |     |     |  |     |     |     |     |  |
| 128                                                                                                                                                                                                                                                                                                                                                                                                                                         | 180                        | 128                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                       | 180                                                        | 180                        |                            |                       |                 |          |           |     |                        |    |     |           |     |                                   |    |     |           |     |                                       |     |     |           |       |                                                    |       |     |           |      |                                                            |      |  |     |        |     |        |  |     |     |     |    |  |     |     |     |     |  |     |     |     |     |  |     |       |     |     |  |     |       |     |     |  |     |     |     |     |  |     |     |     |     |  |     |     |     |     |  |     |       |     |       |  |     |     |     |    |  |     |       |     |     |  |     |       |     |     |  |     |     |     |     |  |     |     |     |     |  |     |     |     |     |  |     |    |     |    |  |     |     |     |     |     |     |    |     |    |  |     |     |     |     |  |     |     |     |     |  |     |     |     |     |  |     |     |     |     |  |
| 581                                                                                                                                                                                                                                                                                                                                                                                                                                         | 40                         | 581                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                       | 40                                                         |                            |                            |                       |                 |          |           |     |                        |    |     |           |     |                                   |    |     |           |     |                                       |     |     |           |       |                                                    |       |     |           |      |                                                            |      |  |     |        |     |        |  |     |     |     |    |  |     |     |     |     |  |     |     |     |     |  |     |       |     |     |  |     |       |     |     |  |     |     |     |     |  |     |     |     |     |  |     |     |     |     |  |     |       |     |       |  |     |     |     |    |  |     |       |     |     |  |     |       |     |     |  |     |     |     |     |  |     |     |     |     |  |     |     |     |     |  |     |    |     |    |  |     |     |     |     |     |     |    |     |    |  |     |     |     |     |  |     |     |     |     |  |     |     |     |     |  |     |     |     |     |  |
| 148                                                                                                                                                                                                                                                                                                                                                                                                                                         | 740                        | 248                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                       | 370                                                        |                            |                            |                       |                 |          |           |     |                        |    |     |           |     |                                   |    |     |           |     |                                       |     |     |           |       |                                                    |       |     |           |      |                                                            |      |  |     |        |     |        |  |     |     |     |    |  |     |     |     |     |  |     |     |     |     |  |     |       |     |     |  |     |       |     |     |  |     |     |     |     |  |     |     |     |     |  |     |     |     |     |  |     |       |     |       |  |     |     |     |    |  |     |       |     |     |  |     |       |     |     |  |     |     |     |     |  |     |     |     |     |  |     |     |     |     |  |     |    |     |    |  |     |     |     |     |     |     |    |     |    |  |     |     |     |     |  |     |     |     |     |  |     |     |     |     |  |     |     |     |     |  |
| 149                                                                                                                                                                                                                                                                                                                                                                                                                                         | 740                        | 249                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                       | 370                                                        |                            |                            |                       |                 |          |           |     |                        |    |     |           |     |                                   |    |     |           |     |                                       |     |     |           |       |                                                    |       |     |           |      |                                                            |      |  |     |        |     |        |  |     |     |     |    |  |     |     |     |     |  |     |     |     |     |  |     |       |     |     |  |     |       |     |     |  |     |     |     |     |  |     |     |     |     |  |     |     |     |     |  |     |       |     |       |  |     |     |     |    |  |     |       |     |     |  |     |       |     |     |  |     |     |     |     |  |     |     |     |     |  |     |     |     |     |  |     |    |     |    |  |     |     |     |     |     |     |    |     |    |  |     |     |     |     |  |     |     |     |     |  |     |     |     |     |  |     |     |     |     |  |
| 178                                                                                                                                                                                                                                                                                                                                                                                                                                         | 740                        | 270                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                       | 370                                                        |                            |                            |                       |                 |          |           |     |                        |    |     |           |     |                                   |    |     |           |     |                                       |     |     |           |       |                                                    |       |     |           |      |                                                            |      |  |     |        |     |        |  |     |     |     |    |  |     |     |     |     |  |     |     |     |     |  |     |       |     |     |  |     |       |     |     |  |     |     |     |     |  |     |     |     |     |  |     |     |     |     |  |     |       |     |       |  |     |     |     |    |  |     |       |     |     |  |     |       |     |     |  |     |     |     |     |  |     |     |     |     |  |     |     |     |     |  |     |    |     |    |  |     |     |     |     |     |     |    |     |    |  |     |     |     |     |  |     |     |     |     |  |     |     |     |     |  |     |     |     |     |  |
| 189                                                                                                                                                                                                                                                                                                                                                                                                                                         | 900                        | 189                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                       | 900                                                        |                            |                            |                       |                 |          |           |     |                        |    |     |           |     |                                   |    |     |           |     |                                       |     |     |           |       |                                                    |       |     |           |      |                                                            |      |  |     |        |     |        |  |     |     |     |    |  |     |     |     |     |  |     |     |     |     |  |     |       |     |     |  |     |       |     |     |  |     |     |     |     |  |     |     |     |     |  |     |     |     |     |  |     |       |     |       |  |     |     |     |    |  |     |       |     |     |  |     |       |     |     |  |     |     |     |     |  |     |     |     |     |  |     |     |     |     |  |     |    |     |    |  |     |     |     |     |     |     |    |     |    |  |     |     |     |     |  |     |     |     |     |  |     |     |     |     |  |     |     |     |     |  |
| Deposit Account Number: <u>Banner &amp; Wilcoff, Ltd.</u><br>Deposit Account Name: <u>19-0733</u>                                                                                                                                                                                                                                                                                                                                           |                            | Fee Description: Surcharge - late filing fee or oath<br>Surcharge - late provisional filing fee or cover sheet<br>Non-English specification<br>For filing a request for reexamination<br>Requesting publication of SIR prior to Examiner action<br>Requesting publication of SIR after Examiner action<br>Extension for reply within first month<br>Extension for reply within second month<br>Extension for reply within third month<br>Extension for reply within fourth month<br>Extension for reply within fifth month<br>Notice of Appeal<br>Filing a brief in support of an appeal<br>Request for oral hearing<br>Petition to institute a public use proceeding<br>Petition to revive - unavoidable<br>Petition to revive - unintentional<br>Utility issue fee (or release)<br>Design issue fee<br>Plant issue fee<br>Petitions to the Commissioner<br>Processing fee under 37 CFR 1.17 (g)<br>Submission of Information Disclosure Sheet<br>Recording each patent assignment per property (times number of properties)<br>Filing a submission after final rejection (37 CFR § 1.129(a))<br>For each additional invention to be examined (37 CFR § 1.129(b))<br>Request for Continued Examination (RCE)<br>Request for expedited examination of a design application                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                |                                                            |                            |                            |                       |                 |          |           |     |                        |    |     |           |     |                                   |    |     |           |     |                                       |     |     |           |       |                                                    |       |     |           |      |                                                            |      |  |     |        |     |        |  |     |     |     |    |  |     |     |     |     |  |     |     |     |     |  |     |       |     |     |  |     |       |     |     |  |     |     |     |     |  |     |     |     |     |  |     |     |     |     |  |     |       |     |       |  |     |     |     |    |  |     |       |     |     |  |     |       |     |     |  |     |     |     |     |  |     |     |     |     |  |     |     |     |     |  |     |    |     |    |  |     |     |     |     |     |     |    |     |    |  |     |     |     |     |  |     |     |     |     |  |     |     |     |     |  |     |     |     |     |  |
| <b>The Commissioner is authorized to: (check all that apply)</b><br><input checked="" type="checkbox"/> Charge fee(s) indicated below <input checked="" type="checkbox"/> Credit any overpayments<br><input checked="" type="checkbox"/> Charge any additional fee(s) during the pendency of this application<br><input type="checkbox"/> Charge fee(s) indicated below, except for the filing fee to the above-identified deposit account. |                            | <b>1. BASIC FILING FEE</b><br><table border="1" style="width: 100%; border-collapse: collapse;"> <thead> <tr> <th>Large Entity Fee Code (\$)</th> <th>Small Entity Fee Code (\$)</th> <th>Fee Description</th> <th>Fee Paid</th> </tr> </thead> <tbody> <tr><td>101</td><td>740 / 201</td><td>370</td><td>Utility filing fee</td><td></td></tr> <tr><td>108</td><td>330 / 208</td><td>165</td><td>Design filing fee</td><td></td></tr> <tr><td>107</td><td>510 / 207</td><td>255</td><td>Plant filing fee</td><td></td></tr> <tr><td>108</td><td>740 / 208</td><td>370</td><td>Reissue filing fee</td><td></td></tr> <tr><td>114</td><td>180 / 214</td><td>80</td><td>Provisional filing fee</td><td></td></tr> </tbody> </table>                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                         |                                                            | Large Entity Fee Code (\$) | Small Entity Fee Code (\$) | Fee Description       | Fee Paid        | 101      | 740 / 201 | 370 | Utility filing fee     |    | 108 | 330 / 208 | 165 | Design filing fee                 |    | 107 | 510 / 207 | 255 | Plant filing fee                      |     | 108 | 740 / 208 | 370   | Reissue filing fee                                 |       | 114 | 180 / 214 | 80   | Provisional filing fee                                     |      |  |     |        |     |        |  |     |     |     |    |  |     |     |     |     |  |     |     |     |     |  |     |       |     |     |  |     |       |     |     |  |     |     |     |     |  |     |     |     |     |  |     |     |     |     |  |     |       |     |       |  |     |     |     |    |  |     |       |     |     |  |     |       |     |     |  |     |     |     |     |  |     |     |     |     |  |     |     |     |     |  |     |    |     |    |  |     |     |     |     |     |     |    |     |    |  |     |     |     |     |  |     |     |     |     |  |     |     |     |     |  |     |     |     |     |  |
| Large Entity Fee Code (\$)                                                                                                                                                                                                                                                                                                                                                                                                                  | Small Entity Fee Code (\$) | Fee Description                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                           | Fee Paid                                                   |                            |                            |                       |                 |          |           |     |                        |    |     |           |     |                                   |    |     |           |     |                                       |     |     |           |       |                                                    |       |     |           |      |                                                            |      |  |     |        |     |        |  |     |     |     |    |  |     |     |     |     |  |     |     |     |     |  |     |       |     |     |  |     |       |     |     |  |     |     |     |     |  |     |     |     |     |  |     |     |     |     |  |     |       |     |       |  |     |     |     |    |  |     |       |     |     |  |     |       |     |     |  |     |     |     |     |  |     |     |     |     |  |     |     |     |     |  |     |    |     |    |  |     |     |     |     |     |     |    |     |    |  |     |     |     |     |  |     |     |     |     |  |     |     |     |     |  |     |     |     |     |  |
| 101                                                                                                                                                                                                                                                                                                                                                                                                                                         | 740 / 201                  | 370                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                       | Utility filing fee                                         |                            |                            |                       |                 |          |           |     |                        |    |     |           |     |                                   |    |     |           |     |                                       |     |     |           |       |                                                    |       |     |           |      |                                                            |      |  |     |        |     |        |  |     |     |     |    |  |     |     |     |     |  |     |     |     |     |  |     |       |     |     |  |     |       |     |     |  |     |     |     |     |  |     |     |     |     |  |     |     |     |     |  |     |       |     |       |  |     |     |     |    |  |     |       |     |     |  |     |       |     |     |  |     |     |     |     |  |     |     |     |     |  |     |     |     |     |  |     |    |     |    |  |     |     |     |     |     |     |    |     |    |  |     |     |     |     |  |     |     |     |     |  |     |     |     |     |  |     |     |     |     |  |
| 108                                                                                                                                                                                                                                                                                                                                                                                                                                         | 330 / 208                  | 165                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                       | Design filing fee                                          |                            |                            |                       |                 |          |           |     |                        |    |     |           |     |                                   |    |     |           |     |                                       |     |     |           |       |                                                    |       |     |           |      |                                                            |      |  |     |        |     |        |  |     |     |     |    |  |     |     |     |     |  |     |     |     |     |  |     |       |     |     |  |     |       |     |     |  |     |     |     |     |  |     |     |     |     |  |     |     |     |     |  |     |       |     |       |  |     |     |     |    |  |     |       |     |     |  |     |       |     |     |  |     |     |     |     |  |     |     |     |     |  |     |     |     |     |  |     |    |     |    |  |     |     |     |     |     |     |    |     |    |  |     |     |     |     |  |     |     |     |     |  |     |     |     |     |  |     |     |     |     |  |
| 107                                                                                                                                                                                                                                                                                                                                                                                                                                         | 510 / 207                  | 255                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                       | Plant filing fee                                           |                            |                            |                       |                 |          |           |     |                        |    |     |           |     |                                   |    |     |           |     |                                       |     |     |           |       |                                                    |       |     |           |      |                                                            |      |  |     |        |     |        |  |     |     |     |    |  |     |     |     |     |  |     |     |     |     |  |     |       |     |     |  |     |       |     |     |  |     |     |     |     |  |     |     |     |     |  |     |     |     |     |  |     |       |     |       |  |     |     |     |    |  |     |       |     |     |  |     |       |     |     |  |     |     |     |     |  |     |     |     |     |  |     |     |     |     |  |     |    |     |    |  |     |     |     |     |     |     |    |     |    |  |     |     |     |     |  |     |     |     |     |  |     |     |     |     |  |     |     |     |     |  |
| 108                                                                                                                                                                                                                                                                                                                                                                                                                                         | 740 / 208                  | 370                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                       | Reissue filing fee                                         |                            |                            |                       |                 |          |           |     |                        |    |     |           |     |                                   |    |     |           |     |                                       |     |     |           |       |                                                    |       |     |           |      |                                                            |      |  |     |        |     |        |  |     |     |     |    |  |     |     |     |     |  |     |     |     |     |  |     |       |     |     |  |     |       |     |     |  |     |     |     |     |  |     |     |     |     |  |     |     |     |     |  |     |       |     |       |  |     |     |     |    |  |     |       |     |     |  |     |       |     |     |  |     |     |     |     |  |     |     |     |     |  |     |     |     |     |  |     |    |     |    |  |     |     |     |     |     |     |    |     |    |  |     |     |     |     |  |     |     |     |     |  |     |     |     |     |  |     |     |     |     |  |
| 114                                                                                                                                                                                                                                                                                                                                                                                                                                         | 180 / 214                  | 80                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                        | Provisional filing fee                                     |                            |                            |                       |                 |          |           |     |                        |    |     |           |     |                                   |    |     |           |     |                                       |     |     |           |       |                                                    |       |     |           |      |                                                            |      |  |     |        |     |        |  |     |     |     |    |  |     |     |     |     |  |     |     |     |     |  |     |       |     |     |  |     |       |     |     |  |     |     |     |     |  |     |     |     |     |  |     |     |     |     |  |     |       |     |       |  |     |     |     |    |  |     |       |     |     |  |     |       |     |     |  |     |     |     |     |  |     |     |     |     |  |     |     |     |     |  |     |    |     |    |  |     |     |     |     |     |     |    |     |    |  |     |     |     |     |  |     |     |     |     |  |     |     |     |     |  |     |     |     |     |  |
| <b>2. EXTRA CLAIM FEES</b><br>Total Claims: <u>20</u> ** = <u>0</u> X Fee from below = <u>0</u><br>Independent Claims: <u>3</u> ** = <u>0</u> X Fee from below = <u>0</u><br>Multiple Dependent: X Fee from below = <u>0</u>                                                                                                                                                                                                                |                            | <b>2. EXTRA CLAIM FEES (continued)</b><br><table border="1" style="width: 100%; border-collapse: collapse;"> <thead> <tr> <th>Large Entity Fee Code (\$)</th> <th>Small Entity Fee Code (\$)</th> <th>Fee Description</th> <th>Fee Paid</th> </tr> </thead> <tbody> <tr><td>103</td><td>18 / 203</td><td>9</td><td>Claims in excess of 20</td><td></td></tr> <tr><td>102</td><td>84 / 202</td><td>42</td><td>Independent claims in excess of 3</td><td></td></tr> <tr><td>104</td><td>280 / 204</td><td>140</td><td>Multiple dependent claim, if not paid</td><td></td></tr> <tr><td>109</td><td>84 / 209</td><td>42</td><td>** Reissue independent claims over original patent</td><td></td></tr> <tr><td>110</td><td>18 / 210</td><td>9</td><td>** Reissue claims in excess of 20 and over original patent</td><td></td></tr> </tbody> </table>                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                         |                                                            | Large Entity Fee Code (\$) | Small Entity Fee Code (\$) | Fee Description       | Fee Paid        | 103      | 18 / 203  | 9   | Claims in excess of 20 |    | 102 | 84 / 202  | 42  | Independent claims in excess of 3 |    | 104 | 280 / 204 | 140 | Multiple dependent claim, if not paid |     | 109 | 84 / 209  | 42    | ** Reissue independent claims over original patent |       | 110 | 18 / 210  | 9    | ** Reissue claims in excess of 20 and over original patent |      |  |     |        |     |        |  |     |     |     |    |  |     |     |     |     |  |     |     |     |     |  |     |       |     |     |  |     |       |     |     |  |     |     |     |     |  |     |     |     |     |  |     |     |     |     |  |     |       |     |       |  |     |     |     |    |  |     |       |     |     |  |     |       |     |     |  |     |     |     |     |  |     |     |     |     |  |     |     |     |     |  |     |    |     |    |  |     |     |     |     |     |     |    |     |    |  |     |     |     |     |  |     |     |     |     |  |     |     |     |     |  |     |     |     |     |  |
| Large Entity Fee Code (\$)                                                                                                                                                                                                                                                                                                                                                                                                                  | Small Entity Fee Code (\$) | Fee Description                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                           | Fee Paid                                                   |                            |                            |                       |                 |          |           |     |                        |    |     |           |     |                                   |    |     |           |     |                                       |     |     |           |       |                                                    |       |     |           |      |                                                            |      |  |     |        |     |        |  |     |     |     |    |  |     |     |     |     |  |     |     |     |     |  |     |       |     |     |  |     |       |     |     |  |     |     |     |     |  |     |     |     |     |  |     |     |     |     |  |     |       |     |       |  |     |     |     |    |  |     |       |     |     |  |     |       |     |     |  |     |     |     |     |  |     |     |     |     |  |     |     |     |     |  |     |    |     |    |  |     |     |     |     |     |     |    |     |    |  |     |     |     |     |  |     |     |     |     |  |     |     |     |     |  |     |     |     |     |  |
| 103                                                                                                                                                                                                                                                                                                                                                                                                                                         | 18 / 203                   | 9                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                         | Claims in excess of 20                                     |                            |                            |                       |                 |          |           |     |                        |    |     |           |     |                                   |    |     |           |     |                                       |     |     |           |       |                                                    |       |     |           |      |                                                            |      |  |     |        |     |        |  |     |     |     |    |  |     |     |     |     |  |     |     |     |     |  |     |       |     |     |  |     |       |     |     |  |     |     |     |     |  |     |     |     |     |  |     |     |     |     |  |     |       |     |       |  |     |     |     |    |  |     |       |     |     |  |     |       |     |     |  |     |     |     |     |  |     |     |     |     |  |     |     |     |     |  |     |    |     |    |  |     |     |     |     |     |     |    |     |    |  |     |     |     |     |  |     |     |     |     |  |     |     |     |     |  |     |     |     |     |  |
| 102                                                                                                                                                                                                                                                                                                                                                                                                                                         | 84 / 202                   | 42                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                        | Independent claims in excess of 3                          |                            |                            |                       |                 |          |           |     |                        |    |     |           |     |                                   |    |     |           |     |                                       |     |     |           |       |                                                    |       |     |           |      |                                                            |      |  |     |        |     |        |  |     |     |     |    |  |     |     |     |     |  |     |     |     |     |  |     |       |     |     |  |     |       |     |     |  |     |     |     |     |  |     |     |     |     |  |     |     |     |     |  |     |       |     |       |  |     |     |     |    |  |     |       |     |     |  |     |       |     |     |  |     |     |     |     |  |     |     |     |     |  |     |     |     |     |  |     |    |     |    |  |     |     |     |     |     |     |    |     |    |  |     |     |     |     |  |     |     |     |     |  |     |     |     |     |  |     |     |     |     |  |
| 104                                                                                                                                                                                                                                                                                                                                                                                                                                         | 280 / 204                  | 140                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                       | Multiple dependent claim, if not paid                      |                            |                            |                       |                 |          |           |     |                        |    |     |           |     |                                   |    |     |           |     |                                       |     |     |           |       |                                                    |       |     |           |      |                                                            |      |  |     |        |     |        |  |     |     |     |    |  |     |     |     |     |  |     |     |     |     |  |     |       |     |     |  |     |       |     |     |  |     |     |     |     |  |     |     |     |     |  |     |     |     |     |  |     |       |     |       |  |     |     |     |    |  |     |       |     |     |  |     |       |     |     |  |     |     |     |     |  |     |     |     |     |  |     |     |     |     |  |     |    |     |    |  |     |     |     |     |     |     |    |     |    |  |     |     |     |     |  |     |     |     |     |  |     |     |     |     |  |     |     |     |     |  |
| 109                                                                                                                                                                                                                                                                                                                                                                                                                                         | 84 / 209                   | 42                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                        | ** Reissue independent claims over original patent         |                            |                            |                       |                 |          |           |     |                        |    |     |           |     |                                   |    |     |           |     |                                       |     |     |           |       |                                                    |       |     |           |      |                                                            |      |  |     |        |     |        |  |     |     |     |    |  |     |     |     |     |  |     |     |     |     |  |     |       |     |     |  |     |       |     |     |  |     |     |     |     |  |     |     |     |     |  |     |     |     |     |  |     |       |     |       |  |     |     |     |    |  |     |       |     |     |  |     |       |     |     |  |     |     |     |     |  |     |     |     |     |  |     |     |     |     |  |     |    |     |    |  |     |     |     |     |     |     |    |     |    |  |     |     |     |     |  |     |     |     |     |  |     |     |     |     |  |     |     |     |     |  |
| 110                                                                                                                                                                                                                                                                                                                                                                                                                                         | 18 / 210                   | 9                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                         | ** Reissue claims in excess of 20 and over original patent |                            |                            |                       |                 |          |           |     |                        |    |     |           |     |                                   |    |     |           |     |                                       |     |     |           |       |                                                    |       |     |           |      |                                                            |      |  |     |        |     |        |  |     |     |     |    |  |     |     |     |     |  |     |     |     |     |  |     |       |     |     |  |     |       |     |     |  |     |     |     |     |  |     |     |     |     |  |     |     |     |     |  |     |       |     |       |  |     |     |     |    |  |     |       |     |     |  |     |       |     |     |  |     |     |     |     |  |     |     |     |     |  |     |     |     |     |  |     |    |     |    |  |     |     |     |     |     |     |    |     |    |  |     |     |     |     |  |     |     |     |     |  |     |     |     |     |  |     |     |     |     |  |
| <b>SUBTOTAL (1)</b> (\$) <u>0</u>                                                                                                                                                                                                                                                                                                                                                                                                           |                            | <b>SUBTOTAL (2)</b> (\$) <u>0</u>                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                         |                                                            |                            |                            |                       |                 |          |           |     |                        |    |     |           |     |                                   |    |     |           |     |                                       |     |     |           |       |                                                    |       |     |           |      |                                                            |      |  |     |        |     |        |  |     |     |     |    |  |     |     |     |     |  |     |     |     |     |  |     |       |     |     |  |     |       |     |     |  |     |     |     |     |  |     |     |     |     |  |     |     |     |     |  |     |       |     |       |  |     |     |     |    |  |     |       |     |     |  |     |       |     |     |  |     |     |     |     |  |     |     |     |     |  |     |     |     |     |  |     |    |     |    |  |     |     |     |     |     |     |    |     |    |  |     |     |     |     |  |     |     |     |     |  |     |     |     |     |  |     |     |     |     |  |
| **or number previously paid, if greater; For Reissues, see above                                                                                                                                                                                                                                                                                                                                                                            |                            | <b>SUBTOTAL (3)</b> (\$) <u>180</u>                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                       |                                                            |                            |                            |                       |                 |          |           |     |                        |    |     |           |     |                                   |    |     |           |     |                                       |     |     |           |       |                                                    |       |     |           |      |                                                            |      |  |     |        |     |        |  |     |     |     |    |  |     |     |     |     |  |     |     |     |     |  |     |       |     |     |  |     |       |     |     |  |     |     |     |     |  |     |     |     |     |  |     |     |     |     |  |     |       |     |       |  |     |     |     |    |  |     |       |     |     |  |     |       |     |     |  |     |     |     |     |  |     |     |     |     |  |     |     |     |     |  |     |    |     |    |  |     |     |     |     |     |     |    |     |    |  |     |     |     |     |  |     |     |     |     |  |     |     |     |     |  |     |     |     |     |  |

| SUBMITTED BY      |                               | Complete (if applicable)        |                  |
|-------------------|-------------------------------|---------------------------------|------------------|
| Name (Print/Type) | Michelle L. Holmes-Son        | Registration No. Attorney/Agent | 47,880           |
| Signature         | <i>Michelle L. Holmes-Son</i> | Telephone                       | 202-508-9100     |
|                   |                               | Date                            | January 23, 2002 |

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Burden Hour Statement: This form is estimated to take 0.2 hours to complete. Time will vary depending upon the needs of the individual case. Any comments on the amount of time you are required to complete this form should be sent to the Chief Information Officer, U.S. Patent and Trademark Office, Washington, DC 20231. **DO NOT SEND FEES OR COMPLETED FORMS TO THIS ADDRESS. SEND TO: Assistant Commissioner for Patents, Washington, DC 20231.**

1656 *AA*

Please type a plus sign (+) inside this box →  Approved for use through 10/31/2002. OMB 0851-0031  
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|                                                                                                    |                        |                        |
|----------------------------------------------------------------------------------------------------|------------------------|------------------------|
| <b>TRANSMITTAL FORM</b><br><small>(to be used for all correspondence after initial filing)</small> | Application Number     | 09/613,826             |
|                                                                                                    | Filing Date            | July 11, 2000          |
|                                                                                                    | First Named Inventor   | Bert Vogelstein et al. |
|                                                                                                    | Group Art Unit         | 1656                   |
|                                                                                                    | Examiner Name          | J. Siew                |
| Total Number of Pages in This Submission                                                           | Attorney Docket Number | 01107.00031            |

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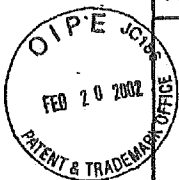
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| ENCLOSURES (check all that apply)                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                       |                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                               |                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                     |
|-------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------|-------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------|-----------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------|
| <input checked="" type="checkbox"/> Fee Transmittal Form<br><input type="checkbox"/> Fee Attached<br><input type="checkbox"/> Amendment / Response<br><input type="checkbox"/> After Final<br><input type="checkbox"/> Affidavits/declaration(s)<br><input type="checkbox"/> Extension of Time Request<br><input type="checkbox"/> Express Abandonment Request<br><input checked="" type="checkbox"/> Information Disclosure Statement<br><input type="checkbox"/> Certified Copy of Priority Document(s)<br><input type="checkbox"/> Response to Missing Parts/ Incomplete Application<br><input type="checkbox"/> Response to Missing Parts under 37 CFR 1.52 or 1.53 | <input type="checkbox"/> Assignment Papers (for an Application)<br><input type="checkbox"/> Drawing(s)<br><input type="checkbox"/> Licensing-related Papers<br><input type="checkbox"/> Petition<br><input type="checkbox"/> Petition to Convert to a Provisional Application<br><input type="checkbox"/> Power of Attorney, Revocation Change of Correspondence Address<br><input type="checkbox"/> Terminal Disclaimer<br><input type="checkbox"/> Request for Refund<br><input type="checkbox"/> CD, Number of CD(s) _____ | <input type="checkbox"/> After Allowance Communication to Group<br><input type="checkbox"/> Appeal Communication to Board of Appeals and Interferences<br><input type="checkbox"/> Appeal Communication to Group (Appeal Notice, Brief, Reply Brief)<br><input type="checkbox"/> Proprietary Information<br><input type="checkbox"/> Status Letter<br><input checked="" type="checkbox"/> Other Enclosure(s) (please identify below):<br><p style="text-align: center;">PTO-1449 (w/references)</p> |
| Remarks                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                 |                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                               |                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                     |

| SIGNATURE OF APPLICANT, ATTORNEY, OR AGENT |                                                 |
|--------------------------------------------|-------------------------------------------------|
| Firm or Individual name                    | Michelle L. Holmes-Son, Registration No. 47,660 |
| Signature                                  | <i>Michelle L. Holmes-Son</i>                   |
| Date                                       | January 23, 2002                                |

| CERTIFICATE OF MAILING                                                                                                                                                                                                             |       |
|------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------|-------|
| I hereby certify that this correspondence is being deposited with the United States Postal Service as first class mail in an envelope addressed to: Assistant Commissioner for Patents, Washington, D.C. 20231 on this date: _____ |       |
| Typed or printed name                                                                                                                                                                                                              | _____ |
| Signature                                                                                                                                                                                                                          | _____ |
| Date                                                                                                                                                                                                                               | _____ |

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|                                                             |                          |                                          |
|-------------------------------------------------------------|--------------------------|------------------------------------------|
| <b>PETITION FOR EXTENSION OF TIME UNDER 37 CFR 1.136(a)</b> |                          | Docket Number (Optional)<br>001107.00031 |
| In re Application of Bert VOGELSTEIN, et a.                 |                          |                                          |
| Application Number 09/613,826                               | Filed July 11, 2000      |                                          |
| For Digital Amplification                                   |                          |                                          |
| Group Art Unit<br>1656                                      | Examiner<br>Jeffrey Siew |                                          |

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

The requested extension and appropriate non-small-entity fee are as follows (check time period desired):

|                                                                    |                  |
|--------------------------------------------------------------------|------------------|
| <input type="checkbox"/> One month (37 CFR 1.17(a)(1))             | \$ _____         |
| <input checked="" type="checkbox"/> Two months (37 CFR 1.17(a)(2)) | \$ <u>400.00</u> |
| <input type="checkbox"/> Three months (37 CFR 1.17(a)(3))          | \$ _____         |
| <input type="checkbox"/> Four months (37 CFR 1.17(a)(4))           | \$ _____         |
| <input type="checkbox"/> Five months (37 CFR 1.17(a)(5))           | \$ _____         |

Applicant claims small entity status. See 37 CFR 1.27. Therefore, the fee amount shown above is reduced by one-half, and the resulting fee is: \$ 200.00.

A check in the amount of the fee is enclosed.

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

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

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

I have enclosed a duplicate copy of this sheet.

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.

attorney or agent under 37 CFR 1.34(a).  
 Registration number if acting under 37 CFR 1.34(a). \_\_\_\_\_

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February 20, 2002  
 \_\_\_\_\_  
 Date

*Michelle L. Holmes*  
 \_\_\_\_\_  
 Signature

Michelle L. Holmes-Son,  
 Reg. No. 47,660  
 \_\_\_\_\_  
 Typed or printed name

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 \_\_\_\_\_ forms are submitted.

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**OIP FEE TRANSMITTAL**  
**for FY 2002**  
 Patent fees are subject to annual revision.

**Completes If Known**

|                      |                         |
|----------------------|-------------------------|
| Application Number   | 09/613,929              |
| Filing Date          | July 11, 2000           |
| First Named Inventor | Bert VOGELSTEIN, et al. |
| Examiner Name        | Jeffrey Siew            |
| Group / Art Unit     | 1658                    |
| Attorney Docket No.  | 001107 00031            |

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**METHOD OF PAYMENT (check all that apply)**

Check  Credit card  Money Order  Other  None

Deposit Account

Deposit Account Number: 19-0733

Deposit Account Name: Berner & Wiscoff, Ltd

The Commissioner is authorized to: (check all that apply)

Charge fee(s) indicated below  Credit any overpayments

Charge any additional fee(s) during the pendency of this application

Charge fee(s) indicated below, except for the filing fee to the above-identified deposit account.

**FEE CALCULATION (continued)**

| Fee Code | Large Entity Fee (\$) | Small Entity Fee (\$) | Fee Description                                        | Fee Paid |
|----------|-----------------------|-----------------------|--------------------------------------------------------|----------|
| 105      | 130                   | 205                   | Surcharge - late filing fee or oath                    |          |
| 127      | 50                    | 227                   | Surcharge - late provisional filing fee or cover sheet |          |
| 139      | 130                   | 130                   | Non-English specification                              |          |
| 147      | 2,520                 | 147                   | For filing a request for reexamination                 |          |
| 112      | 920*                  | 112                   | 920*                                                   |          |
| 113      | 1,840*                | 113                   | 1,840*                                                 |          |
| 115      | 110                   | 215                   | 65                                                     |          |
| 116      | 400                   | 216                   | 200                                                    | 200.00   |
| 117      | 920                   | 217                   | 460                                                    |          |
| 118      | 1,440                 | 218                   | 720                                                    |          |
| 128      | 1,950                 | 228                   | 980                                                    |          |
| 119      | 320                   | 219                   | 160                                                    |          |
| 120      | 320                   | 220                   | 160                                                    |          |
| 121      | 280                   | 221                   | 140                                                    |          |
| 138      | 1,510                 | 138                   | 1,510                                                  |          |
| 140      | 110                   | 240                   | 85                                                     |          |
| 141      | 1,280                 | 241                   | 640                                                    |          |
| 142      | 1,280                 | 242                   | 640                                                    |          |
| 143      | 480                   | 243                   | 240                                                    |          |
| 144      | 820                   | 244                   | 310                                                    |          |
| 122      | 130                   | 122                   | 130                                                    |          |
| 123      | 50                    | 123                   | 50                                                     |          |
| 126      | 180                   | 126                   | 180                                                    |          |
| 581      | 40                    | 581                   | 40                                                     |          |
| 146      | 740                   | 246                   | 370                                                    |          |
| 149      | 740                   | 249                   | 370                                                    |          |
| 179      | 740                   | 279                   | 370                                                    |          |
| 169      | 600                   | 169                   | 600                                                    |          |

Other fee (specify) \_\_\_\_\_

\*Reduced by Basic Filing Fee Paid

**SUBTOTAL (3)** (\$) 200

**FEE CALCULATION**

**1. BASIC FILING FEE**

| Large Entity Fee Code (\$) | Small Entity Fee Code (\$) | Fee Description | Fee Paid               |
|----------------------------|----------------------------|-----------------|------------------------|
| 101                        | 201                        | 370             | Utility filing fee     |
| 100                        | 206                        | 185             | Design filing fee      |
| 107                        | 207                        | 255             | Plant filing fee       |
| 108                        | 208                        | 370             | Reissue filing fee     |
| 114                        | 214                        | 60              | Provisional filing fee |

**SUBTOTAL (1)** (\$) 0

**2. EXTRA CLAIM FEES**

Total Claims: 20 \*\* = 0

Independent Claims: 3 \*\* = 0

Multiple Dependent: X = 0

| Large Entity Fee Code (\$) | Small Entity Fee Code (\$) | Fee Description | Fee Paid                                                   |
|----------------------------|----------------------------|-----------------|------------------------------------------------------------|
| 103                        | 203                        | 0               | Claims in excess of 20                                     |
| 102                        | 202                        | 42              | Independent claims in excess of 3                          |
| 104                        | 204                        | 140             | Multiple dependent claim, if not paid                      |
| 109                        | 209                        | 42              | ** Reissue independent claims over original patent         |
| 110                        | 210                        | 0               | ** Reissue claims in excess of 20 and over original patent |

**SUBTOTAL (2)** (\$) 0

\*\*or number previously paid, if greater; For Reissues, see above

**SUBMITTED BY** (Complete if applicable)

|                   |                        |                                 |        |           |                   |
|-------------------|------------------------|---------------------------------|--------|-----------|-------------------|
| Name (Print/Type) | Michelle L. Holmes-Son | Registration No. Attorney/Agent | 47,860 | Telephone | (202) 508-9100    |
| Signature         |                        |                                 |        | Date      | February 20, 2002 |

**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-2036.

**Burden Hour Statement:** This form is estimated to take 0.2 hours to complete. Time will vary depending upon the needs of the individual case. Any comments on the amount of time you are required to complete this form should be sent to the Chief Information Officer, U.S. Patent and Trademark Office, Washington, DC 20231. **DO NOT SEND FEES OR COMPLETED FORMS TO THIS ADDRESS. SEND TO:** Assistant Commissioner for Patents, Washington, DC 20231



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PTO/SB/21 (08-00)  
Approved for use through 10/31/2002. OMB 0651-0024  
U.S. Patent and Trademark Office; U.S. DEPARTMENT OF COMMERCE

AF/1656/8  
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|                                                                                            |                        |                         |
|--------------------------------------------------------------------------------------------|------------------------|-------------------------|
| <b>TRANSMITTAL FORM</b><br><i>(to be used for all correspondence after initial filing)</i> | Application Number     | 09/613,826              |
|                                                                                            | Filing Date            | July 11, 2000           |
|                                                                                            | First Named Inventor   | Bert VOGELSTEIN, et al. |
|                                                                                            | Group Art Unit         | 1656                    |
|                                                                                            | Examiner Name          | Jeffrey Siew            |
| Total Number of Pages In This Submission                                                   | Attorney Docket Number | 001107.00031            |

| ENCLOSURES (check all that apply)                                                                                                                                                                                                                                                                                                                                                                       |                                                                                                                                                                                                                                                                                                                                                                                                                        |                                                                                                                                                                                                                                                                                                                                                    |
|---------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------|------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------|----------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------|
| <input checked="" type="checkbox"/> Fee Transmittal Form<br><input type="checkbox"/> Fee Attached                                                                                                                                                                                                                                                                                                       | <input type="checkbox"/> Assignment Papers (for an Application)<br><input type="checkbox"/> Drawing(s)                                                                                                                                                                                                                                                                                                                 | <input type="checkbox"/> After Allowance Communication to Group<br><input type="checkbox"/> Appeal Communication to Board of Appeals and Interferences<br><input type="checkbox"/> Appeal Communication to Group (Appeal Notice, Brief, Reply Brief)<br><input type="checkbox"/> Proprietary Information<br><input type="checkbox"/> Status Letter |
| <input checked="" type="checkbox"/> Amendment / Response<br><input checked="" type="checkbox"/> After Final<br><input type="checkbox"/> Affidavits/declaration(s)                                                                                                                                                                                                                                       | <input type="checkbox"/> Licensing-related Papers<br><input type="checkbox"/> Petition<br><input type="checkbox"/> Petition to Convert to a Provisional Application<br><input type="checkbox"/> Power of Attorney, Revocation<br>Change of Correspondence Address<br><input type="checkbox"/> Terminal Disclaimer<br><input type="checkbox"/> Request for Refund<br><input type="checkbox"/> CD, Number of CD(s) _____ | <input checked="" type="checkbox"/> Other Enclosure(s) (please identify below):<br><b>Sequence Listing</b><br><b>Computer Readable Disk</b>                                                                                                                                                                                                        |
| <input type="checkbox"/> Extension of Time Request<br><input type="checkbox"/> Express Abandonment Request<br><input type="checkbox"/> Information Disclosure Statement<br><input type="checkbox"/> Certified Copy of Priority Document(s)<br><input type="checkbox"/> Response to Missing Parts/Incomplete Application<br><input type="checkbox"/> Response to Missing Parts under 37 CFR 1.52 or 1.53 | Remarks                                                                                                                                                                                                                                                                                                                                                                                                                |                                                                                                                                                                                                                                                                                                                                                    |

| SIGNATURE OF APPLICANT, ATTORNEY, OR AGENT |                                         |
|--------------------------------------------|-----------------------------------------|
| Firm or Individual name                    | Michelle L. Holmes-Son, Reg. No. 47,860 |
| Signature                                  | <i>Michelle L. Holmes-Son</i>           |
| Date                                       | February 20, 2002                       |

| CERTIFICATE OF MAILING                                                                                                                                                                                                             |       |
|------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------|-------|
| I hereby certify that this correspondence is being deposited with the United States Postal Service as first class mail in an envelope addressed to: Assistant Commissioner for Patents, Washington, D.C. 20231 on this date: _____ |       |
| Typed or printed name                                                                                                                                                                                                              | _____ |
| Signature                                                                                                                                                                                                                          | _____ |
| Date                                                                                                                                                                                                                               | _____ |

Burden Hour Statement: This form is estimated to take 0.2 hours to complete. Time will vary upon the needs of the individual case. Any comments on the amount of time you are required to complete this form should be sent to the Chief Information Officer, U.S. Patent and Trademark Office, Washington, DC 20231. DO NOT SEND FEES OR COMPLETED FORMS TO THIS ADDRESS. SEND TO: Assistant Commissioner for Patents, Washington, DC 20231.



PATENT

#175  
2/20/02  
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FEB 25 2002  
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IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re Application of )  
 Bert Vogelstein, et. al. ) Group Art Unit: 1656  
 Serial No. 09/613,826 )  
 Filing Date: July 11, 2000 ) Examiner: J Siew  
 ) Box AF  
 ) Docket No. 01107.00031

For: **DIGITAL AMPLIFICATION**

ENTERED  
3/22/02  
mjk

AMENDMENT AFTER FINAL REJECTION

Assistant Commissioner for Patents  
Washington, D.C. 20231

Sir:

In response to the Final Office Action mailed September 20, 2001, applicants request entry of the following amendments. Claims 1-64 are pending in the application and are allowed. A petition for a two-month extension of time is enclosed herewith. No other fees are believed due to make this response filed timely. If any additional fee is due please change our Deposit Account No. 19-0733.

ok to  
file  
3/19/02

IN THE CLAIMS

Please cancel claims 65-69.



SEQUENCE LISTING

Please replace the sequence listing in the application with the accompanying substitute sequence listing. A computer readable form and paper copy of the substitute sequence listing are enclosed. They are believed to be identical in content. The substitute sequence listing introduces no new matter.

IN THE SPECIFICATION

The paragraph beginning page 4, line 19.

---

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).

The paragraph beginning page 5, line 10.

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.

C2

The paragraph beginning page 5, line 17.

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 K1 (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.

C3

110

## REMARKS

### The Amendments

The specification has been amended to enter a substitute sequence listing. The substitute sequence listing includes sequences that were not present in the prior sequence listing. The additional sequences are disclosed in Figures 2, 4, and 5 of the drawings.

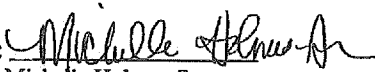
The paragraph beginning at page 4, line 19 has been amended to disclose the sequence identifier of each of the wildtype or mutant *ras* sequences shown in Figure 2.

The paragraph beginning at page 5, line 10 and the paragraph beginning at page 5, line 17 have each been amended to disclose the sequence identifier for each of the wildtype or mutant *ras* sequences shown in Figures 4 and 5, respectively.

These amendments were not made earlier as applicants only first became aware of this oversight after the final rejection was mailed. Rejected claims 65-69 have been canceled without prejudice to their future prosecution in continuation applications. A notice of allowance is respectfully requested.

Respectfully submitted,

Date: February 20, 2002

By:   
Michelle Holmes-Son  
Registration No. 47,660

Banner & Witcoff, Ltd.  
1001 G Street, NW  
Washington, DC 20001  
202-508-9100

MARKED UP VERSION TO SHOW CHANGES MADE

The paragraph beginning page 4, line 19.

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).

The paragraph beginning page 5, line 10.

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.

The paragraph beginning page 5, line 17.

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  $1.0 \pm 0.1$  (mean  $\pm$  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 K1 (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.



107.31.ST25  
SEQUENCE LISTING

<110> Vogelstein, Bert  
Kinzler, Kenneth W.

<120> DIGITAL AMPLIFICATION

<130> 01107.00031

<140> 09/613,826  
<141> 2000-07-11

<150> US 60/146,792  
<151> 1999-08-02

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2/15

107.31.ST25

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;)k-



107.31.ST25

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251

|                          |                                      |                                          |  |
|--------------------------|--------------------------------------|------------------------------------------|--|
| <b>Interview Summary</b> | <b>Application No.</b><br>09/613,828 | <b>Applicant(s)</b><br>VOGELSTEIN ET AL. |  |
|                          | <b>Examiner</b><br>Jeffrey Slew      | <b>Art Unit</b><br>1656                  |  |

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

(1) Jeffrey Slew (3) \_\_\_\_\_

(2) Michelle Holmes-Son (4) \_\_\_\_\_

Date of Interview: 04 March 2002.

Type: a)  Telephonic b)  Video Conference  
c)  Personal [copy given to: 1)  applicant 2)  applicant's representative]

Exhibit shown or demonstration conducted: d)  Yes e)  No.  
If Yes, brief description: \_\_\_\_\_.

Claim(s) discussed: 1-64.

Identification of prior art discussed: \_\_\_\_\_.

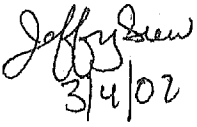
Agreement with respect to the claims f)  was reached. g)  was not reached. h)  N/A.

Substance of Interview including description of the general nature of what was agreed to if an agreement was reached, or any other comments: discussed newly disclosed prior art.

(A fuller description, if necessary, and a copy of the amendments which the examiner agreed would render the claims allowable, if available, must be attached. Also, where no copy of the amendments that would render the claims allowable is available, a summary thereof must be attached.)

i)  It is not necessary for applicant to provide a separate record of the substance of the interview (if box is checked).

Unless the paragraph above has been checked, THE FORMAL WRITTEN REPLY TO THE LAST OFFICE ACTION MUST INCLUDE THE SUBSTANCE OF THE INTERVIEW. (See MPEP Section 713.04). If a reply to the last Office action has already been filed, APPLICANT IS GIVEN ONE MONTH FROM THIS INTERVIEW DATE TO FILE A STATEMENT OF THE SUBSTANCE OF THE INTERVIEW. See Summary of Record of Interview requirements on reverse side or on attached sheet.

  
3/4/02

Examiner Note: You must sign this form unless it is an Attachment to a signed Office action. \_\_\_\_\_  
Examiner's signature, if required

#19

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1637

RAW SEQUENCE LISTING  
PATENT APPLICATION: US/09/613,826A  
DATE: 03/01/2002  
TIME: 15:29:37

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Output Set: N:\CRF3\03012002\I613826A.raw

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4 Kinzler, Kenneth W.  
6 <120> TITLE OF INVENTION: DIGITAL AMPLIFICATION  
8 <130> FILE REFERENCE: 01107.00031  
10 <140> CURRENT APPLICATION NUMBER: 09/613,826A  
11 <141> CURRENT FILING DATE: 2000-07-11  
13 <150> PRIOR APPLICATION NUMBER: US 60/146,792  
14 <151> PRIOR FILING DATE: 1999-08-02  
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RECEIVED  
MAR 14 2002  
TECH CENTER 1600/2900

26

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24





VERIFICATION SUMMARY                      DATE: 03/01/2002  
PATENT APPLICATION: US/09/613,826A        TIME: 15:29:38

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## File History Report

Paper number \_\_\_\_\_ is missing from the United States Patent Trademark Office's copy of the file History. No additional information is available.

The following page(s) 5 of 5 of paper number 19 is/are missing from the United States Patent and Trademark Office's original copy of the file history. No additional information is available

The following checked item(s) below of paper number \_\_\_\_\_ is/are missing from the United States Patent and Trademark Office's original copy of the file history. No additional information is available

- PTO 1449
- PTO 892
- PTO 948
- PTO 1474
- Assignment
- Cover page

Additional comments: \_\_\_\_\_

|                          |                 |                  |  |
|--------------------------|-----------------|------------------|--|
| <b>Interview Summary</b> | Application No. | Applicant(s)     |  |
|                          | 09/613,829      | TAKESHITA ET AL. |  |
|                          | Examiner        | Art Unit         |  |
|                          | Jeffrey Slew    | 1856             |  |

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

(1) Jeffrey Slew. (3) \_\_\_\_\_.

(2) Michelle Holmes Son. (4) \_\_\_\_\_.

Date of Interview: 19 March 2002.

Type: a)  Telephonic b)  Video Conference  
c)  Personal [copy given to: 1)  applicant 2)  applicant's representative]

Exhibit shown or demonstration conducted: d)  Yes e)  No.  
If Yes, brief description: \_\_\_\_\_.

Claim(s) discussed: 1-64.

Identification of prior art discussed: \_\_\_\_\_.

Agreement with respect to the claims f)  was reached. g)  was not reached. h)  N/A.

Substance of interview including description of the general nature of what was agreed to if an agreement was reached, or any other comments: discussed that newly cited prior art do not read on the prior art. For example, Halford while performing dilutions and amplifications, the claimed invention is performing a dilution which results in a first number of samples which contain selected genetic sequence and second number of assay samples which contain a reference and comparing to ascertain a ratio.

(A fuller description, if necessary, and a copy of the amendments which the examiner agreed would render the claims allowable, if available, must be attached. Also, where no copy of the amendments that would render the claims allowable is available, a summary thereof must be attached.)

i)  It is not necessary for applicant to provide a separate record of the substance of the interview (if box is checked).

Unless the paragraph above has been checked, THE FORMAL WRITTEN REPLY TO THE LAST OFFICE ACTION MUST INCLUDE THE SUBSTANCE OF THE INTERVIEW. (See MPEP Section 713.04). If a reply to the last Office action has already been filed, APPLICANT IS GIVEN ONE MONTH FROM THIS INTERVIEW DATE TO FILE A STATEMENT OF THE SUBSTANCE OF THE INTERVIEW. See Summary of Record of Interview requirements on reverse side or on attached sheet.

Examiner Note: You must sign this form unless it is an Attachment to a signed Office action.

\_\_\_\_\_  
Examiner's signature, if required



|                               |                 |                  |  |
|-------------------------------|-----------------|------------------|--|
| <b>Notice of Allowability</b> | Application No. | Applicant(s)     |  |
|                               | 09/813,829      | TAKESHITA ET AL. |  |
|                               | Examiner        | Art Unit         |  |
|                               | Jeffrey Slew    | 1656             |  |

**-- 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 int. 3/19/02.
2.  The allowed claim(s) is/are 1-64.
3.  The drawings filed on 11 July 2000 are accepted by the Examiner.
4.  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 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: \_\_\_\_\_
5.  Acknowledgment is made of a claim for domestic priority under 35 U.S.C. § 119(e) (to a provisional application).
  - (a)  The translation of the foreign language provisional application has been received.
6.  Acknowledgment is made of a claim for domestic priority under 35 U.S.C. §§ 120 and/or 121.

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.**

7.  A SUBSTITUTE OATH OR DECLARATION must be submitted. Note the attached EXAMINER'S AMENDMENT or NOTICE OF INFORMAL PATENT APPLICATION (PTO-152) which gives reason(s) why the oath or declaration is deficient.
8.  CORRECTED DRAWINGS must be submitted.
  - (a)  including changes required by the Notice of Draftperson's Patent Drawing Review (PTO-948) attached
    - 1)  hereto or 2)  to Paper No. \_\_\_\_\_.
  - (b)  including changes required by the proposed drawing correction filed \_\_\_\_\_, which has been approved by the Examiner.
  - (c)  including changes required by the attached Examiner's Amendment / Comment or in the Office action of Paper No. \_\_\_\_\_.

Identifying Indicia such as the application number (see 37 CFR 1.84(c)) should be written on the drawings in the top margin (not the back) of each sheet. The drawings should be filed as a separate paper with a transmittal letter addressed to the Official Draftperson.
9.  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)**

|                                                                                                                                                                                                                                                                                                                                                                                                                                            |                                                                                                                                                                                                                                                                                                                                                                                                                                         |
|--------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------|-----------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------|
| <ol style="list-style-type: none"> <li>1 <input type="checkbox"/> Notice of References Cited (PTO-892)</li> <li>3 <input checked="" type="checkbox"/> Notice of Draftperson's Patent Drawing Review (PTO-948)</li> <li>6 <input type="checkbox"/> Information Disclosure Statements (PTO-1449), Paper No. _____</li> <li>7 <input type="checkbox"/> Examiner's Comment Regarding Requirement for Deposit of Biological Material</li> </ol> | <ol style="list-style-type: none"> <li>2 <input type="checkbox"/> Notice of Informal Patent Application (PTO-152)</li> <li>4 <input checked="" type="checkbox"/> Interview Summary (PTO-413), Paper No. <u>18522</u></li> <li>5 <input type="checkbox"/> Examiner's Amendment/Comment</li> <li>8 <input checked="" type="checkbox"/> Examiner's Statement of Reasons for Allowance</li> <li>9 <input type="checkbox"/> Other</li> </ol> |
|--------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------|-----------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------|

#### REASONS FOR ALLOWANCE

1. The following is an examiner's statement of reasons for allowance:

Claims 33-37 are allowable. There is no prior art that teach or suggest a molecular beacon probe that has a loop consisting of 16 base pairs and having a  $T_m$  of 50-51C and the stem consisting of CACG sequence. The closest prior art is Tyagi et al (US6,037,130) teach molecular beacon with a stem comprising CACG (see col. 11 probe 3) but with a loop of  $T_m$  50C. Moreover, the prior art has been focused on the  $T_m$  of the stem which relates to the functioning of the opening and closing of the hairpin during hybridization.

Claims 1-32 & 38-64 is allowable. There is no prior art that teach or suggest diluting a nucleic acid template in a sample to a plurality of sample and amplifying the template molecule in the samples and analyzing amplified molecules to determine the first number of samples containing the selected genetic sequence and second number assay samples which contain a reference genetic sequence and comparing the two numbers. Moreover, there is no prior art that teach or suggest that one tenth or one fiftieth of samples in a set comprise N molecules such that  $1/N$  is larger than the ratio of selected genetic sequence to total genetic sequences required for the step of analyzing to determine presence of selected genetic sequence. The closest prior art is Lapidus et al who teach a reference and target nucleic acid amplification and concentration determination. However, his determination of concentration is within a sample and they do not teach or suggest a dilution.

Application/Control Number: 09/613,829  
Art Unit: 1656

Page 3

Any comments considered necessary by applicant must be submitted no later than the payment of the issue fee and, to avoid processing delays, should preferably accompany the issue fee. Such submissions should be clearly labeled "Comments on Statement of Reasons for Allowance."

#### CONCLUSION

2. Any inquiry concerning this communication or earlier communications from the examiner should be directed to Jeffrey Siew whose telephone number is (703) 305-3886 and whose e-mail address is Jeffrey.Siew@uspto.gov. However, the office cannot guarantee security through the e-mail system nor should official papers be transmitted through this route. The examiner is on flex-time schedule and can best be reached on weekdays from 6:30 a.m. to 3 p.m. If attempts to reach the examiner are unsuccessful, the examiner's supervisor, Gary Benzion, can be reached on (703)-308-1119.

Any inquiry of a general nature or relating to the status of this application or proceeding should be directed to the receptionist for Technology Center 1600 whose telephone number is (703) 308-0196.

Papers related to this application may be submitted to Group 1600 by facsimile transmission. Papers should be faxed to Group 1600 via the PTO Fax Center located in Crystal

Application/Control Number: 09/613,829  
Art Unit: 1656

Page 4

**Mall 1.** The faxing of such papers must conform with the notice published in the Official Gazette, 1096 OG 30 (November 15, 1989). The CM1 Center numbers for Group 1600 are Voice (703) 308-3290 and Before Final FAX (703) 872-9306 or After Final FAX (703) 30872-9307.

  
Jeffrey Siew

March 19, 2002



UNITED STATES PATENT AND TRADEMARK OFFICE

UNITED STATES DEPARTMENT OF COMMERCE
United States Patent and Trademark Office
Address: COMMISSIONER OF PATENTS AND TRADEMARKS
Washington, D.C. 20593
www.uspto.gov

NOTICE OF ALLOWANCE AND FEE(S) DUE

22907 7590 03/24/2002
BANNER & WITCOFF
1001 G STREET N W
SUITE 1100
WASHINGTON, DC 20001

EXAMINER
SIEW, JEFFREY

ART UNIT CLASS-SUBCLASS
1637 435-006600

DATE MAILED: 03/24/2002

Table with 5 columns: APPLICATION NO., FILING DATE, FIRST NAMED INVENTOR, ATTORNEY DOCKET NO., CONFIRMATION NO.
09/613,826 07/11/2000 Bert Vogelstein 01107 00031 9893

TITLE OF INVENTION: DIGITAL AMPLIFICATION

Table with 7 columns: TOTAL CLAIMS, APPLN. TYPE, SMALL ENTITY, ISSUE FEE, PUBLICATION FEE, TOTAL FEE(S) DUE, DATE DUE
64 nonprovisional YES \$640 \$0 \$640 06/24/2002

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 REFLECTS A CREDIT FOR ANY PREVIOUSLY PAID ISSUE FEE APPLIED IN THIS APPLICATION. THE PTOL-85B (OR AN EQUIVALENT) MUST BE RETURNED WITHIN THIS PERIOD EVEN IF NO FEE IS DUE OR THE APPLICATION WILL BE REGARDED AS ABANDONED.

HOW TO REPLY TO THIS NOTICE:

- I. Review the SMALL ENTITY status shown above. If the SMALL ENTITY is shown as YES, verify your current SMALL ENTITY status:
A. If the status is changed, pay the PUBLICATION FEE (if required) and twice the amount of the ISSUE FEE shown above and notify the United States Patent and Trademark Office of the change in status, or
B. If the status is the same, pay the TOTAL FEE(S) DUE shown above.

If the SMALL ENTITY is shown as NO:

- A. Pay TOTAL FEE(S) DUE shown above, or
B. If applicant claimed SMALL ENTITY status before, or is now claiming SMALL ENTITY status, check the box below and enclose the PUBLICATION FEE and 1/2 the ISSUE FEE shown above.
[ ] Applicant claims SMALL ENTITY status. See 37 CFR 1.27.

II. PART B - FEE(S) TRANSMITTAL should be completed and returned to the United States Patent and Trademark Office (USPTO) with your ISSUE FEE and PUBLICATION FEE (if required). Even if the fee(s) have already been paid, Part B - Fee(s) Transmittal should be completed and returned. If you are charging the fee(s) to your deposit account, section "4b" of Part B - Fee(s) Transmittal should be completed and an extra copy of the form should be submitted.

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

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

**PART B - FEE(S) TRANSMITTAL**

Complete and mail this form, together with applicable fee(s), to: **Box ISSUE FEE  
Assistant Commissioner for Patents  
Washington, D.C. 20231**

**MAILING INSTRUCTIONS.** This form should be used for transmitting the **ISSUE FEE** and **PUBLICATION FEE** (if required). Blocks 1 through 4 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 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: Legibly mark-up with any corrections or use Block 1)

22907 7390 03/24/2002

**BANNER & WITCOFF  
1001 G STREET N W  
SUITE 1100  
WASHINGTON, DC 20001**

Note: The certificate of mailing below 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.

**Certificate of Mailing**

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 Box Issue Fee address above on the date indicated below.

|                             |
|-----------------------------|
| _____<br>(Depositor's name) |
| _____<br>(Signature)        |
| _____<br>(Date)             |

| APPLICATION NO. | FILING DATE | FIRST NAMED INVENTOR | ATTORNEY DOCKET NO. | CONFIRMATION NO. |
|-----------------|-------------|----------------------|---------------------|------------------|
| 09/613,826      | 07/11/2000  | Bert Vogelstein      | 01107.00031         | 9893             |

TITLE OF INVENTION: DIGITAL AMPLIFICATION

| TOTAL CLAIMS | APPLN. TYPE    | SMALL ENTITY | ISSUE FEE | PUBLICATION FEE | TOTAL FEE(S) DUE | DATE DUE   |
|--------------|----------------|--------------|-----------|-----------------|------------------|------------|
| 64           | nonprovisional | YES          | \$640     | \$0             | \$640            | 06/24/2002 |

| EXAMINER      | ART UNIT | CLASS-SUBCLASS |
|---------------|----------|----------------|
| SIEW, JEFFREY | 1637     | 435-006000     |

- |                                                                                                                                                                                                                                                                                                                                                                                                                     |                                                                                                                                                                                                                                                                                                                                                                                       |
|---------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------|---------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------|
| <p>1. Change of correspondence address or indication of "Fee Address" (37 CFR 1.363). Use of PTO form(s) and Customer Number are recommended, but not required.</p> <p><input type="checkbox"/> Change of correspondence address (or Change of Correspondence Address form PTO/SB/122) attached.</p> <p><input type="checkbox"/> "Fee Address" indication (or "Fee Address" Indication form PTO/SB/47) attached</p> | <p>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.</p> <p>1 _____</p> <p>2 _____</p> <p>3 _____</p> |
|---------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------|---------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------|

**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. Inclusion of assignee data is only appropriate when an assignment has been previously submitted to the USPTO or is being submitted under separate cover. 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

- |                                                                                                                                                                                                                  |                                                                                                                                                                                                                                                                                                                                                                                                                |
|------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------|----------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------|
| <p><b>4a. The following fee(s) are enclosed:</b></p> <p><input type="checkbox"/> Issue Fee</p> <p><input type="checkbox"/> Publication Fee</p> <p><input type="checkbox"/> Advance Order - # of Copies _____</p> | <p><b>4b. Payment of Fee(s):</b></p> <p><input type="checkbox"/> A check in the amount of the fee(s) is enclosed.</p> <p><input type="checkbox"/> Payment by credit card. Form PTO-2038 is attached.</p> <p><input type="checkbox"/> The Commissioner is hereby authorized by charge the required fee(s), or credit any overpayment, to Deposit Account Number _____ (enclose an extra copy of this form).</p> |
|------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------|----------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------|

The COMMISSIONER OF PATENTS AND TRADEMARKS is requested to apply the Issue Fee and Publication Fee (if any) or to re-apply any previously paid issue fee in the application identified above.

(Authorized Signature) \_\_\_\_\_ (Date) \_\_\_\_\_

NOTE: The Issue Fee and Publication Fee (if required) will not be accepted from anyone other than the applicant; a registered attorney or agent; or the assignee or other party in interest as shown by the records of the United States Patent and Trademark Office.

**Burden Hour Statement:** This form is estimated to take 0.2 hours to complete. Time will vary depending on the needs of the individual case. Any comments on the amount of time required to complete this form should be sent to the Chief Information Officer, United States Patent and Trademark Office, Washington, D.C. 20231. DO NOT SEND FEES OR COMPLETE/FID FORMS TO THIS ADDRESS. SEND FEES AND THIS FORM TO: Box Issue Fee, Assistant Commissioner for Patents, Washington, D.C. 20231

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UNITED STATES PATENT AND TRADEMARK OFFICE

UNITED STATES DEPARTMENT OF COMMERCE  
United States Patent and Trademark Office  
Address: COMMUNICATIONS PATENT AND TRADEMARKS  
Washington, D.C. 20231  
www.uspto.gov

| APPLICATION NO                                                              | FILING DATE | FIRST NAMED INVENTOR | ATTORNEY DOCKET NO. | CONFIRMATION NO |
|-----------------------------------------------------------------------------|-------------|----------------------|---------------------|-----------------|
| 09/613,826                                                                  | 07/11/2000  | Bert Vogelstein      | 01107.00031         | 9893            |
| 22907                                                                       | 7390        | 03/24/2002           | EXAMINER            |                 |
| BANNER & WITCOFF<br>1001 G STREET N W<br>SUITE 1100<br>WASHINGTON, DC 20001 |             |                      | SIEW, JEFFREY       |                 |
|                                                                             |             |                      | ART UNIT            | PAPER NUMBER    |
|                                                                             |             |                      | 1637                | 21              |
| DATE MAILED: 03/24/2002                                                     |             |                      |                     |                 |

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

The patent term adjustment to date is 0 days. If the issue fee is paid on the date that is three months after the mailing date of this notice and the patent issues on the Tuesday before the date that is 28 weeks (six and a half months) after the mailing date of this notice, the term adjustment will be 0 days.

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) system. (<http://pair.uspto.gov>)

*Supplemental*  
Notice of Allowability

|                 |                  |  |
|-----------------|------------------|--|
| Application No. | Applicant(s)     |  |
| 09/613,829      | TAKESHITA ET AL. |  |
| Examiner        | Art Unit         |  |
| Jeffrey Slew    | 1656             |  |

-- 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 Int 3/19/02.
2.  The allowed claim(s) is/are 1-64.
3.  The drawings filed on 11 July 2000 are accepted by the Examiner.
4.  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 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: \_\_\_\_\_.
5.  Acknowledgment is made of a claim for domestic priority under 35 U.S.C. § 119(e) (to a provisional application).
  - (a)  The translation of the foreign language provisional application has been received.
6.  Acknowledgment is made of a claim for domestic priority under 35 U.S.C. §§ 120 and/or 121.

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.

7.  A SUBSTITUTE OATH OR DECLARATION must be submitted. Note the attached EXAMINER'S AMENDMENT or NOTICE OF INFORMAL PATENT APPLICATION (PTO-152) which gives reason(s) why the oath or declaration is deficient.
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Attachment(s)

- |                                                                                                      |                                                                                        |
|------------------------------------------------------------------------------------------------------|----------------------------------------------------------------------------------------|
| <input type="checkbox"/> Notice of References Cited (PTO-892)                                        | <input type="checkbox"/> Notice of Informal Patent Application (PTO-152)               |
| <input checked="" type="checkbox"/> Notice of Draftsperson's Patent Drawing Review (PTO-948)         | <input checked="" type="checkbox"/> Interview Summary (PTO-413), Paper No. <u>1822</u> |
| <input type="checkbox"/> Information Disclosure Statements (PTO-1449), Paper No. _____               | <input type="checkbox"/> Examiner's Amendment/Comment                                  |
| <input type="checkbox"/> Examiner's Comment Regarding Requirement for Deposit of Biological Material | <input checked="" type="checkbox"/> Examiner's Statement of Reasons for Allowance      |
|                                                                                                      | <input type="checkbox"/> Other                                                         |



### REASONS FOR ALLOWANCE

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Application/Control Number: 09/613,829

Page 4

Art Unit: 1656

**Mall 1.** The faxing of such papers must conform with the notice published in the Official Gazette, 1096 OG 30 (November 15, 1989). The CM1 Center numbers for Group 1600 are Voice (703) 308-3290 and Before Final FAX (703) 872-9306 or After Final FAX (703) 30872-9307.

  
Jeffrey Siew

March 19, 2002

## File History Report

Paper number \_\_\_\_\_ is missing from the United States Patent Trademark Office's copy of the file History. No additional information is available.

The following page(s) PTO - 948 of paper number 22 is/are missing from the United States Patent and Trademark Office's original copy of the file history. No additional information is available

The following checked item(s) below of paper number \_\_\_\_\_ is/are missing from the United States Patent and Trademark Office's original copy of the file history. No additional information is available

- PTO 1449
- PTO 892
- PTO 948
- PTO 1474
- Assignment
- Cover page

Additional comments: \_\_\_\_\_

\* Corrected Copy \*



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NOTICE OF ALLOWANCE AND FEE(S) DUE

22907 7590 03/26/2002  
BANNER & WITCOFF  
1001 G STREET N W  
SUITE 1100  
WASHINGTON, DC 20001

EXAMINER

SIEW, JEFFREY

ART UNIT CLASS-SUBCLASS

1637 435-006000

DATE MAILED: 03/26/2002

| APPLICATION NO. | FILING DATE | FIRST NAMED INVENTOR | ATTORNEY DOCKET NO. | CONFIRMATION NO |
|-----------------|-------------|----------------------|---------------------|-----------------|
| 09/613,826      | 07/11/2000  | Bert Vogelstein      | 01107,00031         | 9893            |

TITLE OF INVENTION, DIGITAL AMPLIFICATION

| TOTAL CLAIMS | APPLN. TYPE    | SMALL ENTITY | ISSUE FEE | PUBLICATION FEE | TOTAL FEE(S) DUE | DATE DUE   |
|--------------|----------------|--------------|-----------|-----------------|------------------|------------|
| 64           | nonprovisional | YES          | \$640     | \$0             | \$640            | 06/26/2002 |

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

| APPLICATION NO | FILING DATE | FIRST NAMED INVENTOR | ATTORNEY DOCKET NO | CONFIRMATION NO |
|----------------|-------------|----------------------|--------------------|-----------------|
| 09/613,826     | 07/11/2000  | Bert Vogelstein      | 01107.00031        | 9893            |

TITLE OF INVENTION: DIGITAL AMPLIFICATION

| TOTAL CLAIMS | APPLN. TYPE    | SMALL ENTITY | ISSUE FEE | PUBLICATION FEE | TOTAL FEE(S) DUE | DATE DUE   |
|--------------|----------------|--------------|-----------|-----------------|------------------|------------|
| 64           | nonprovisional | YES          | \$640     | \$0             | \$640            | 06/26/2002 |

| EXAMINER      | ART UNIT | CLASS-SUBCLASS |
|---------------|----------|----------------|
| STEW, JEFFREY | 1637     | 435-006000     |

1. Change of correspondence address or indication of "Fee Address" (37 CFR 1.363). Use of PTO form(s) and Customer Number are recommended, but not required.

- Change of correspondence address (or Change of Correspondence Address form PTO/SB/122) attached.
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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. Inclusion of assignee data is only appropriate when an assignment has been previously submitted to the USPTO or is being submitted under separate cover. 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

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|                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                     |        |
|-----------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------|--------|
| (Authorized Signature)                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                              | (Date) |
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*Supplemental*

| APPLICATION NO.                                                             | FILING DATE | FIRST NAMED INVENTOR | ATTORNEY DOCKET NO. | CONFIRMATION NO. |
|-----------------------------------------------------------------------------|-------------|----------------------|---------------------|------------------|
| 09/613,826                                                                  | 07/11/2000  | Bert Vogelstein      | 01107.00031         | 9893             |
| 22907                                                                       | 7590        | 03/26/2002           | EXAMINER            |                  |
| BANNER & WITCOFF<br>1001 G STREET N W<br>SUITE 1100<br>WASHINGTON, DC 20001 |             |                      | SIEW, JEFFREY       |                  |
|                                                                             |             |                      | ART UNIT            | PAPER NUMBER     |
|                                                                             |             |                      | 1637                | 22               |
| DATE MAILED: 03/26/2002                                                     |             |                      |                     |                  |

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

The patent term adjustment to date is 0 days. If the issue fee is paid on the date that is three months after the mailing date of this notice and the patent issues on the Tuesday before the date that is 28 weeks (six and a half months) after the mailing date of this notice, the term adjustment will be 0 days.

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) system. (<http://pair.uspto.gov>)



PART B - FEE(S) TRANSMITTAL

Complete and mail this form, together with applicable fee(s), to:

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MAILING INSTRUCTIONS: This form should be used for transmitting the ISSUE FEE and PUBLICATION FEE (if required). Blocks 1 through 4 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.

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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 Box Issue Fee address above on the date indicated below.

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Signature)  
(Date)

Table with 5 columns: APPLICATION NO., FILING DATE, FIRST NAMED INVENTOR, ATTORNEY DOCKET NO., CONFIRMATION NO.

TITLE OF INVENTION: DIGITAL AMPLIFICATION

Table with 7 columns: TOTAL CLAIMS, APPLN. TYPE, SMALL ENTITY, ISSUE FEE, PUBLICATION FEE, TOTAL FEE(S) DUE, DATE DUE

Table with 3 columns: EXAMINER, ART UNIT, CLASS-SUBCLASS

- 1. Change of correspondence address or indication of "Fee Address" (37 CFR 1.363). Use of PTO form(s) and Customer Number are recommended, but not 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.

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. Inclusion of assignee data is only appropriate when an assignment has been previously submitted to the USPTO or is being submitted under separate cover. Completion of this form is NOT a substitute for filing an assignment.
(A) NAME OF ASSIGNEE: Johns Hopkins University
(B) RESIDENCE: (CITY and STATE OR COUNTRY): Baltimore, Maryland

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

- 4a. The following Fee(s) are enclosed:
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[ ] Publication Fee
[X] Advance Order - # of Copies 10
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(Authorized Signature) Michelle Holmes-Son, Reg. No. 47,660 May 21, 2002

05/22/2002 TREASURER 00000060 150733 0561200E

01 FC:PAF 640.00 EN
02 FC:361 30.00 EN

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PATENT APPLICATION

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In Re Application of: )  
Bert VOGELSTEIN et al. )  
Serial No. 09/613,826 ) Group Art Unit: 1656  
Filed: July 11, 2000 ) Examiner: J. Siew  
For: DIGITAL AMPLIFICATION ) Box: AF  
Attorney Docket No. 01107.00031

INFORMATION DISCLOSURE STATEMENT

Assistant Commissioner for Patents  
Washington, D.C. 20231

Sir:

Pursuant to 37 C.F.R. § 1.56 and in compliance with 37 C.F.R. § 1.97(d), Applicants herewith submit Form PTO 1449 listing references for consideration in connection with the above-identified application. A copy of each reference is provided herewith.

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.

04/15/02 00:14 FAX

005/007


Serial No. 09/613,826

Applicants respectfully request that the Examiner consider and enter these documents into the file of the above-identified application. A fee of \$180.00 as set forth in 37 C.F.R. § 1.17(p) is enclosed herewith to ensure consideration and entry of the cited documents by the Examiner. If any additional fee is deemed necessary, the Commissioner is authorized to charge our Deposit Account No. 19-0733.

Respectfully submitted,

Date: January 23, 2002

By:

  
Michelle L. Holmes-Son  
Registration No. 47,660

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1001 G Street, N.W.  
Eleventh Floor  
Washington, D.C. 20001-4597  
(202) 508-9100

- 2 -

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| <b>INFORMATION DISCLOSURE STATEMENT BY APPLICANT</b><br><br>(use as many sheets as necessary) |                        | Application Number       | 09/613,826             |
|                                                                                               |                        | Filing Date              | July 11, 2000          |
|                                                                                               |                        | First Named Inventor     | Bert Vogelstein et al. |
|                                                                                               |                        | Group Art Unit           | 1856                   |
|                                                                                               |                        | Examiner Name            | J. Shaw                |
| Sheet 2 of 2                                                                                  | Attorney Docket Number | D1107.00031              |                        |

| OTHER PRIOR ART - NON PATENT LITERATURE DOCUMENTS |                       |                                                                                                                                                                                                                                                                 |                |
|---------------------------------------------------|-----------------------|-----------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------|----------------|
| Examiner Initials *                               | Cite No. <sup>1</sup> | Include name of the author (in CAPITAL LETTERS), title of the article (when appropriate), title of the item (book, magazine, journal, serial, symposium, catalog, etc.), date, page(s), volume-issue number(s), publisher, city and/or country where published. | T <sup>2</sup> |
| JS                                                |                       | A. PIATEK et al., "Molecular Beacon Sequence Analysis for Detecting Drug Resistance in Mycobacterium Tuberculosis", <i>Nature Biotechnology</i> , April 1999, pp. 359-363, Vol. 18, No. 4                                                                       |                |
|                                                   |                       | S. TYAGI et al., "Multicolor Molecular Beacons for allele discrimination", <i>Nature Biotechnology</i> , pp. 303-308, January 1998, Vol. 16, No. 1                                                                                                              |                |
|                                                   |                       | J. A. J. VET et al., "Multicolor Detection of Four Pathogenic Retroviruses Using Molecular Beacons", <i>Proceedings of the National Academy of Sciences of the United States</i> , May 25, 1999, pp. 6394-6398, Vol. 96, No. 11                                 |                |
|                                                   |                       | S. TYAGI et al., "Molecular Beacons: probes that Fluoresce Upon Hybridization", <i>Nature Biotechnology</i> , 1996, pp. 303-308, Vol. 14, No. 3                                                                                                                 |                |
|                                                   |                       | W. P. HALFORD et al., "The Inherent Quantitative Capacity of the Reverse Transcription-Polymerase Chain Reaction", <i>Analytical Biochemistry</i> , January 15, 1989, pp. 101-109, Vol. 268, No. 2                                                              |                |
|                                                   |                       | B. VOGELSTEIN et al., "Digital PCR", <i>Proceedings of the National Academy of Sciences of the United States</i> , August 3, 1988, pp. 8228-8231, Vol. 85, No. 16                                                                                               |                |
| JS                                                |                       | K. D. E. EVERETT et al., "Identification of nine species of the Chlamydiaceae Using PCR-RFLP", April 1999, pp. 803-812, Vol. 49, No. 2                                                                                                                          |                |
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|                    |                |                 |         |
|--------------------|----------------|-----------------|---------|
| Examiner Signature | <i>J. Shaw</i> | Date Considered | 4/15/02 |
|--------------------|----------------|-----------------|---------|

\* EXAMINER: Initial if reference considered, whether or not citation is in conformance with MPEP 808. Draw line through citation if not in conformance and not considered. Include copy of this form with next communication to applicant.

<sup>1</sup> Unique citation designation number (optional). <sup>2</sup> Applicant is to place a check mark here if English language Translation is attached.

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## FEE TRANSMITTAL for FY 2002

Patent fees are subject to annual revision.

|                                         |                         |                          |               |
|-----------------------------------------|-------------------------|--------------------------|---------------|
| <b>TOTAL AMOUNT OF PAYMENT (\$)</b> 180 |                         | <b>Complete if Known</b> |               |
| Application Number                      | 09/013,826              | Filing Date              | July 11, 2000 |
| First Named Inventor                    | Eert Vogelestein et al. | Examiner Name            | J. Siew       |
| Group / Art Unit                        | 1958                    | Attorney Docket No.      | 01107.00031   |

| <p><b>METHOD OF PAYMENT (check all that apply)</b></p> <p><input type="checkbox"/> Check <input type="checkbox"/> Credit card <input type="checkbox"/> Money <input type="checkbox"/> Other <input type="checkbox"/> None<br/>Order</p> <p><input checked="" type="checkbox"/> Deposit Account:</p> <p>Deposit Account Number: <span style="border: 1px solid black; padding: 2px;">Banner B Warrant, Ltd.</span></p> <p>Deposit Account Name: <span style="border: 1px solid black; padding: 2px;">10-0735</span></p> <p>The Commissioner is authorized to: (check all that apply)<br/> <input checked="" type="checkbox"/> Charge fee(s) indicated below <input type="checkbox"/> Credit any overpayments<br/> <input type="checkbox"/> Charge any additional fee(s) during the pendency of this application<br/> <input type="checkbox"/> Charge fee(s) indicated below, except for the filing fee to the above-identified deposit account.</p> <p style="text-align: center;"><b>FEE CALCULATION</b></p> <p><b>1. BASIC FILING FEE</b></p> <table border="1" style="width: 100%; border-collapse: collapse; font-size: x-small;"> <thead> <tr> <th>Large Entity Code</th> <th>Small Entity Code</th> <th>Fee (\$)</th> <th>Fee Description</th> <th>Fee Paid</th> </tr> </thead> <tbody> <tr><td>101</td><td>201</td><td>370</td><td>Utility filing fee</td><td></td></tr> <tr><td>105</td><td>205</td><td>185</td><td>Design filing fee</td><td></td></tr> <tr><td>107</td><td>207</td><td>250</td><td>Plant filing fee</td><td></td></tr> <tr><td>108</td><td>208</td><td>370</td><td>Reissue filing fee</td><td></td></tr> <tr><td>114</td><td>214</td><td>80</td><td>Provisional filing fee</td><td></td></tr> <tr><td colspan="4" style="text-align: right;"><b>SUBTOTAL (1)</b></td><td style="border: 1px solid black; text-align: center;">180</td></tr> </tbody> </table> <p><b>2. EXTRA CLAIM FEES</b></p> <p>Total Claims: 20 <input type="checkbox"/> = 0 <input type="checkbox"/> X <input type="checkbox"/> = 0 <input type="checkbox"/><br/>         Independent Claims: 3 <input type="checkbox"/> = 0 <input type="checkbox"/> X <input type="checkbox"/> = 0 <input type="checkbox"/><br/>         Multiple Dependents: <input type="checkbox"/> X <input type="checkbox"/> = 0 <input type="checkbox"/></p> <table border="1" style="width: 100%; border-collapse: collapse; font-size: x-small;"> <thead> <tr> <th>Large Entity Code</th> <th>Small Entity Code</th> <th>Fee (\$)</th> <th>Fee Description</th> <th>Fee Paid</th> </tr> </thead> <tbody> <tr><td>103</td><td>203</td><td>9</td><td>Claims in excess of 20</td><td></td></tr> <tr><td>102</td><td>202</td><td>42</td><td>Independent claims in excess of 3</td><td></td></tr> <tr><td>104</td><td>204</td><td>180</td><td>Multiple dependent claim, if not paid</td><td></td></tr> <tr><td>109</td><td>209</td><td>42</td><td>Reissue independent claims over original patent</td><td></td></tr> <tr><td>110</td><td>210</td><td>0</td><td>Reissue claims in excess of 20 and over original patent</td><td></td></tr> <tr><td colspan="4" style="text-align: right;"><b>SUBTOTAL (2)</b></td><td style="border: 1px solid black; text-align: center;">0</td></tr> </tbody> </table> <p><b>3. ADDITIONAL FEE</b></p> <table border="1" style="width: 100%; border-collapse: collapse; font-size: x-small;"> <thead> <tr> <th>Large Entity Code</th> <th>Small Entity Code</th> <th>Fee (\$)</th> <th>Fee Description</th> <th>Fee Paid</th> </tr> </thead> <tbody> <tr><td>109</td><td>209</td><td>65</td><td>Surcharge - late filing fee or cost</td><td></td></tr> <tr><td>127</td><td>227</td><td>28</td><td>Surcharge - late provisional filing fee or cover sheet</td><td></td></tr> <tr><td>138</td><td>238</td><td>150</td><td>Non-English specification</td><td></td></tr> <tr><td>137</td><td>237</td><td>2,820</td><td>For filing a request for reconsideration</td><td></td></tr> <tr><td>112</td><td>212</td><td>620*</td><td>Requesting publication of OIR prior to Examiner action</td><td></td></tr> <tr><td>113</td><td>213</td><td>1,840*</td><td>Requesting publication of SIR after Examiner action</td><td></td></tr> <tr><td>115</td><td>215</td><td>65</td><td>Extension for reply within first month</td><td></td></tr> <tr><td>116</td><td>216</td><td>200</td><td>Extension for reply within second month</td><td></td></tr> <tr><td>117</td><td>217</td><td>400</td><td>Extension for reply within third month</td><td></td></tr> <tr><td>118</td><td>218</td><td>720</td><td>Extension for reply within fourth month</td><td></td></tr> <tr><td>126</td><td>226</td><td>680</td><td>Extension for reply within fifth month</td><td></td></tr> <tr><td>110</td><td>210</td><td>150</td><td>Negotiate of Appeal</td><td></td></tr> <tr><td>120</td><td>220</td><td>150</td><td>Filing a brief in support of an appeal</td><td></td></tr> <tr><td>121</td><td>221</td><td>140</td><td>Request for oral hearing</td><td></td></tr> <tr><td>135</td><td>235</td><td>1,510</td><td>Petition to institute a public use proceeding</td><td></td></tr> <tr><td>140</td><td>240</td><td>85</td><td>Petition to revive - unavoidable</td><td></td></tr> <tr><td>161</td><td>261</td><td>840</td><td>Petition to revive - unintentional</td><td></td></tr> <tr><td>142</td><td>242</td><td>840</td><td>Utility issue fee (or release)</td><td></td></tr> <tr><td>143</td><td>243</td><td>230</td><td>Design issue fee</td><td></td></tr> <tr><td>144</td><td>244</td><td>310</td><td>Plant issue fee</td><td></td></tr> <tr><td>182</td><td>282</td><td>130</td><td>Petitions to the Commissioner</td><td></td></tr> <tr><td>123</td><td>223</td><td>60</td><td>Processing fee under 37 CFR 1.17 (c)</td><td></td></tr> <tr><td>128</td><td>228</td><td>180</td><td>Submission of Information Disclosure Sheet</td><td>180</td></tr> <tr><td>691</td><td>691</td><td>40</td><td>Recording each patent assignment per disparity (times number of properties)</td><td></td></tr> <tr><td>146</td><td>246</td><td>370</td><td>Filing a submission after final rejection (37 CFR § 1.125(a))</td><td></td></tr> <tr><td>148</td><td>248</td><td>370</td><td>For each additional invention to be examined (37 CFR § 1.125(b))</td><td></td></tr> <tr><td>176</td><td>276</td><td>870</td><td>Request for Continued Examination (RCE)</td><td></td></tr> <tr><td>183</td><td>283</td><td>600</td><td>Request for expedited examination of a design application</td><td></td></tr> <tr><td colspan="4" style="text-align: right;"><b>SUBTOTAL (3)</b></td><td style="border: 1px solid black; text-align: center;">180</td></tr> </tbody> </table> <p>*Reduced by Basic Filing Fee Paid</p> <p><b>SUBTOTAL (4)</b> <span style="border: 1px solid black; padding: 2px;">(\$)</span> 180</p> | Large Entity Code             | Small Entity Code               | Fee (\$)                                                                    | Fee Description | Fee Paid     | 101 | 201 | 370 | Utility filing fee |  | 105 | 205 | 185 | Design filing fee |  | 107 | 207 | 250 | Plant filing fee |  | 108 | 208 | 370 | Reissue filing fee |  | 114 | 214 | 80 | Provisional filing fee |  | <b>SUBTOTAL (1)</b> |  |  |  | 180 | Large Entity Code | Small Entity Code | Fee (\$) | Fee Description | Fee Paid | 103 | 203 | 9 | Claims in excess of 20 |  | 102 | 202 | 42 | Independent claims in excess of 3 |  | 104 | 204 | 180 | Multiple dependent claim, if not paid |  | 109 | 209 | 42 | Reissue independent claims over original patent |  | 110 | 210 | 0 | Reissue claims in excess of 20 and over original patent |  | <b>SUBTOTAL (2)</b> |  |  |  | 0 | Large Entity Code | Small Entity Code | Fee (\$) | Fee Description | Fee Paid | 109 | 209 | 65 | Surcharge - late filing fee or cost |  | 127 | 227 | 28 | Surcharge - late provisional filing fee or cover sheet |  | 138 | 238 | 150 | Non-English specification |  | 137 | 237 | 2,820 | For filing a request for reconsideration |  | 112 | 212 | 620* | Requesting publication of OIR prior to Examiner action |  | 113 | 213 | 1,840* | Requesting publication of SIR after Examiner action |  | 115 | 215 | 65 | Extension for reply within first month |  | 116 | 216 | 200 | Extension for reply within second month |  | 117 | 217 | 400 | Extension for reply within third month |  | 118 | 218 | 720 | Extension for reply within fourth month |  | 126 | 226 | 680 | Extension for reply within fifth month |  | 110 | 210 | 150 | Negotiate of Appeal |  | 120 | 220 | 150 | Filing a brief in support of an appeal |  | 121 | 221 | 140 | Request for oral hearing |  | 135 | 235 | 1,510 | Petition to institute a public use proceeding |  | 140 | 240 | 85 | Petition to revive - unavoidable |  | 161 | 261 | 840 | Petition to revive - unintentional |  | 142 | 242 | 840 | Utility issue fee (or release) |  | 143 | 243 | 230 | Design issue fee |  | 144 | 244 | 310 | Plant issue fee |  | 182 | 282 | 130 | Petitions to the Commissioner |  | 123 | 223 | 60 | Processing fee under 37 CFR 1.17 (c) |  | 128 | 228 | 180 | Submission of Information Disclosure Sheet | 180 | 691 | 691 | 40 | Recording each patent assignment per disparity (times number of properties) |  | 146 | 246 | 370 | Filing a submission after final rejection (37 CFR § 1.125(a)) |  | 148 | 248 | 370 | For each additional invention to be examined (37 CFR § 1.125(b)) |  | 176 | 276 | 870 | Request for Continued Examination (RCE) |  | 183 | 283 | 600 | Request for expedited examination of a design application |  | <b>SUBTOTAL (3)</b> |  |  |  | 180 | <p><b>4. SUBMITTED BY</b></p> <table border="1" style="width: 100%; border-collapse: collapse;"> <tr> <td>Name (Print/Type)</td> <td>Michelle L. Holmes-Son</td> <td>Registration No. Attorney/Agent</td> <td>47,600</td> <td>Telephone</td> <td>202-502-9100</td> </tr> <tr> <td>Signature</td> <td><i>Michelle L. Holmes-Son</i></td> <td>Date</td> <td>January 23, 2002</td> <td colspan="2"></td> </tr> </table> <p style="font-size: x-small; text-align: center;">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-2058.</p> <p style="font-size: x-small;">Burden Hour Statement: This form is estimated to take 0.2 hours to complete. This will vary depending upon the needs of the individual case. Any comments on the amount of time you are required to complete this form should be sent to the Chief Information Officer, U.S. Patent and Trademark Office, Washington, DC 20231. DO NOT SEND FEES OR COMPLETED FORMS TO THIS ADDRESS. SEND TO: Assistant Commissioner for Patents, Washington, DC 20231.</p> | Name (Print/Type) | Michelle L. Holmes-Son | Registration No. Attorney/Agent | 47,600 | Telephone | 202-502-9100 | Signature | <i>Michelle L. Holmes-Son</i> | Date | January 23, 2002 |  |  |
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|                     |  |  |  |     |                   |                   |          |                 |          |     |     |   |                        |  |     |     |    |                                   |  |     |     |     |                                       |  |     |     |    |                                                 |  |     |     |   |                                                         |  |                     |  |  |  |   |                   |                   |          |                 |          |     |     |    |                                     |  |     |     |    |                                                        |  |     |     |     |                           |  |     |     |       |                                          |  |     |     |      |                                                        |  |     |     |        |                                                     |  |     |     |    |                                    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|                     |  |  |  |     |                   |                   |          |                 |          |     |     |   |                        |  |     |     |    |                                   |  |     |     |     |                                       |  |     |     |    |                                                 |  |     |     |   |                                                         |  |                     |  |  |  |   |                   |                   |          |                 |          |     |     |    |                                     |  |     |     |    |                                                        |  |     |     |     |                           |  |     |     |       |                                          |  |     |     |      |                                                        |  |     |     |        |                                                     |  |     |     |    |                                    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|                     |  |  |  |     |                   |                   |          |                 |          |     |     |   |                        |  |     |     |    |                                   |  |     |     |     |                                       |  |     |     |    |                                                 |  |     |     |   |                                                         |  |                     |  |  |  |   |                   |                   |          |                 |          |     |     |    |                                     |  |     |     |    |                                                        |  |     |     |     |                           |  |     |     |       |                                          |  |     |     |      |                                                        |  |     |     |        |                                                     |  |     |     |    |                                    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|                     |  |  |  |     |                   |                   |          |                 |          |     |     |   |                        |  |     |     |    |                                   |  |     |     |     |                                       |  |     |     |    |                                                 |  |     |     |   |                                                         |  |                     |  |  |  |   |                   |                   |          |                 |          |     |     |    |                                     |  |     |     |    |                                                        |  |     |     |     |                           |  |     |     |       |                                          |  |     |     |      |                                                        |  |     |     |        |                                                     |  |     |     |    |                                    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                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                      | Michelle L. Holmes-Son        | Registration No. Attorney/Agent | 47,600                                                                      | Telephone       | 202-502-9100 |     |     |     |                    |  |     |     |     |                   |  |     |     |     |                  |  |     |     |     |                    |  |     |     |    |                        |  |                     |  |  |  |     |                   |                   |          |                 |          |     |     |   |                        |  |     |     |    |                                   |  |     |     |     |                                       |  |     |     |    |                                                 |  |     |     |   |                                                         |  |                     |  |  |  |   |                   |                   |          |                 |          |     |     |    |                                     |  |     |     |    |                                                        |  |     |     |     |                           |  |     |     |       |                                          |  |     |     |      |                                                        |  |     |     |        |                                                     |  |     |     |    |                                        |  |     |     |     |                                         |  |     |     |     |                                        |  |     |     |     |                                         |  |     |     |     |                                        |  |     |     |     |                     |  |     |     |     |                                        |  |     |     |     |                          |  |     |     |       |                                               |  |     |     |    |                                  |  |     |     |     |                                    |  |     |     |     |                                |  |     |     |     |                  |  |     |     |     |                 |  |     |     |     |                               |  |     |     |    |                                      |  |     |     |     |                                            |     |     |     |    |                                                                             |  |     |     |     |                                                               |  |     |     |     |                                                                  |  |     |     |     |                                         |  |     |     |     |                                                           |  |                     |  |  |  |     |                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                         |                   |                        |                                 |        |           |              |           |                               |      |                  |  |  |
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                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                      | <i>Michelle L. Holmes-Son</i> | Date                            | January 23, 2002                                                            |                 |              |     |     |     |                    |  |     |     |     |                   |  |     |     |     |                  |  |     |     |     |                    |  |     |     |    |                        |  |                     |  |  |  |     |                   |                   |          |                 |          |     |     |   |                        |  |     |     |    |                                   |  |     |     |     |                                       |  |     |     |    |                                                 |  |     |     |   |                                                         |  |                     |  |  |  |   |                   |                   |          |                 |          |     |     |    |                                     |  |     |     |    |                                                        |  |     |     |     |                           |  |     |     |       |                                          |  |     |     |      |                                                        |  |     |     |        |                                                     |  |     |     |    |                                        |  |     |     |     |                                         |  |     |     |     |                                        |  |     |     |     |                                         |  |     |     |     |                                        |  |     |     |     |                     |  |     |     |     |                                        |  |     |     |     |                          |  |     |     |       |                                               |  |     |     |    |                                  |  |     |     |     |                                    |  |     |     |     |                                |  |     |     |     |                  |  |     |     |     |                 |  |     |     |     |                               |  |     |     |    |                                      |  |     |     |     |                                            |     |     |     |    |                                                                             |  |     |     |     |                                                               |  |     |     |     |                                                                  |  |     |     |     |                                         |  |     |     |     |                                                           |  |                     |  |  |  |     |                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                         |                   |                        |                                 |        |           |              |           |                               |      |                  |  |  |

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|                                                                                            | Filing Date            | July 11, 2000          |
|                                                                                            | First Named Inventor   | Bert Vogelstein et al. |
|                                                                                            | Group Art Unit         | 1656                   |
|                                                                                            | Examiner Name          | J. Siew                |
| Total Number of Pages in This Submission                                                   | Attorney Docket Number | 01107.00031            |

| ENCLOSURES (check all that apply)                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                      |                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                               |                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                    |
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| Firm or Individual name                    | Michelle L. Holmes-Son, Registration No. 47,880 |
| Signature                                  |                                                 |
| Date                                       | January 23, 2002                                |

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| <b>COMPANY:</b><br>United States Patent and Trademark Office | <b>DATE:</b><br>April 15, 2002                              |
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| <b>RE:</b><br>U.S. Serial Number: 09/613,826                 | <b>OUR REFERENCE NO.:</b><br>01107.00031                    |

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**NAME:** Michelle L. Holmes-Son      **PHONE:** 1-202-508-9220

**COMMENTS:**

Pursuant to our telephone conversation of this morning, attached please find a copy of our information disclosure statement filed January 23, 2002. Please return by fax after you are satisfied with your review of the document.

Yours truly,

  
Michelle L. Holmes-Son (47.660)

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IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re Application of )  
Bert Vogelstein, et. al. ) Group Art Unit: 1637  
Serial No. 09/613,826 ) Examiner: Jeffrey Siew  
Filing Date: July 11, 2000 ) Docket No. 01107.00031

For: DIGITAL AMPLIFICATION

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Attached hereto for filing in the United States Patent and Trademark Office are seven (7) sheets of formal drawings (FIGS. 1 A-C, 2-5) for the above-identified patent application.

Please charge any fee in connection with the filing of these drawings to our Deposit Account No. 19-0733 and forward the recorded document to the undersigned. A duplicate of this sheet is enclosed.

Respectfully submitted,

Date: May 21, 2002

By: Michelle L. Holmes-Son  
Michelle L. Holmes-Son  
Registration No. 47,660

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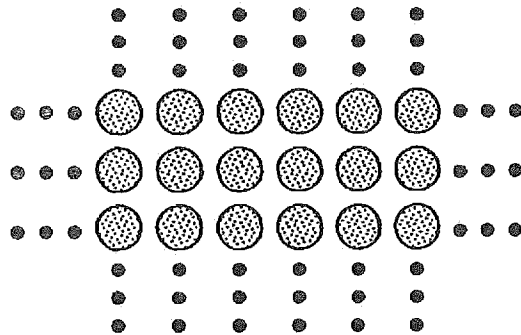
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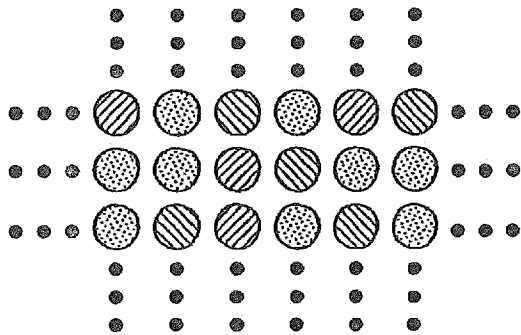
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FIG. 1A

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WELL PCR



STEP 2 ↓ ADD FLUORESCENT PROBES  
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-  = NO PCR PRODUCT
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**FIG. 1B**

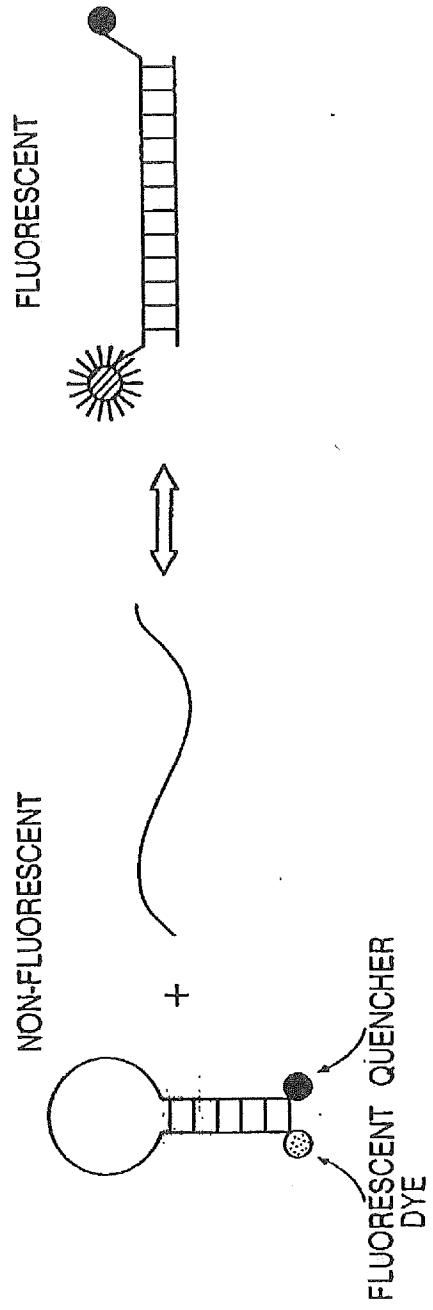


FIG. 1C

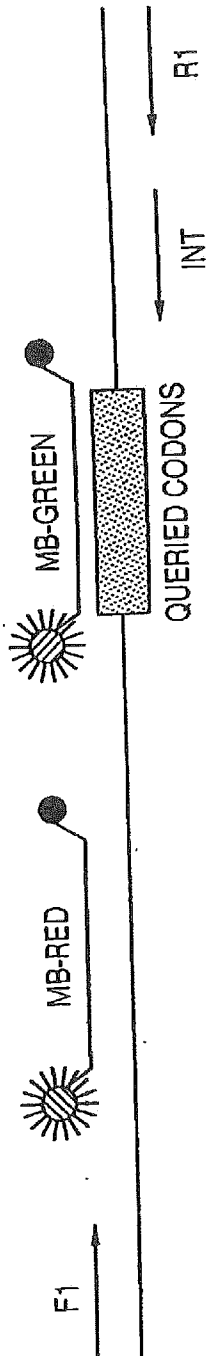
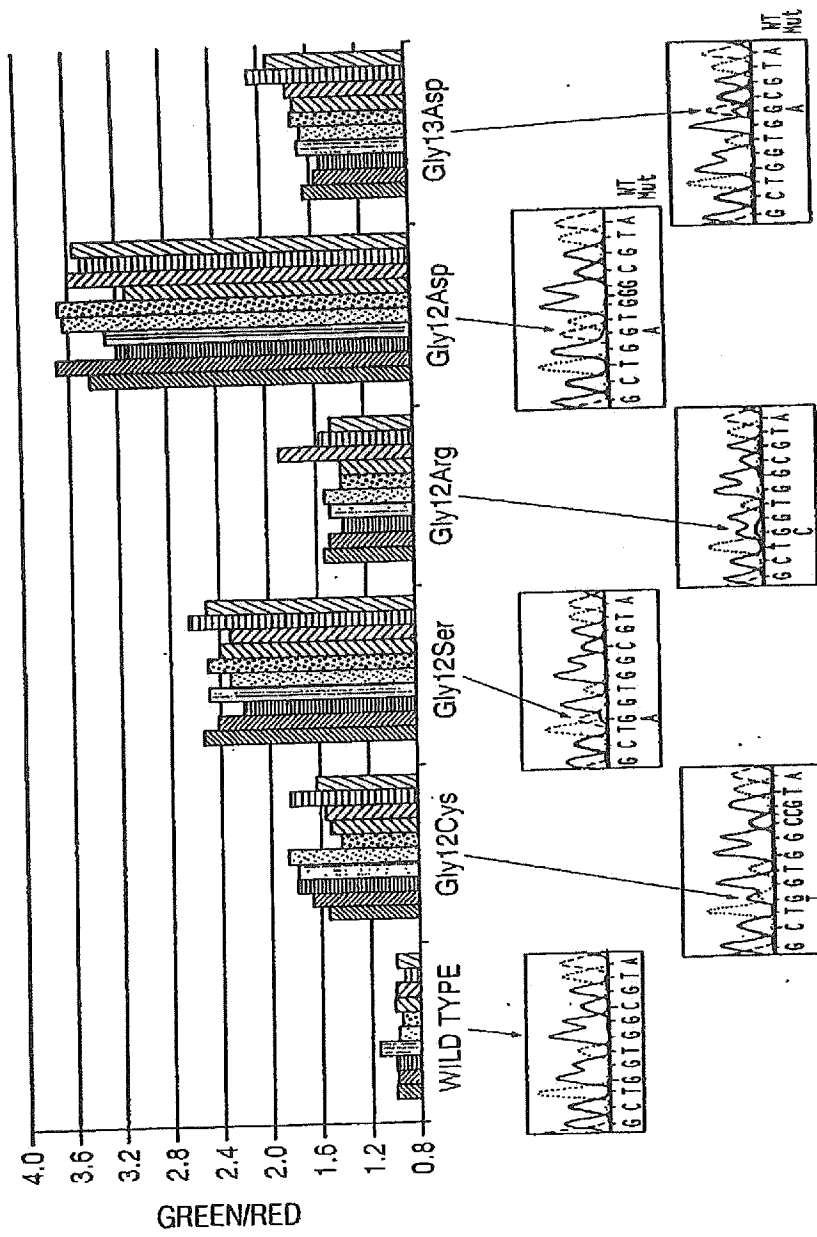
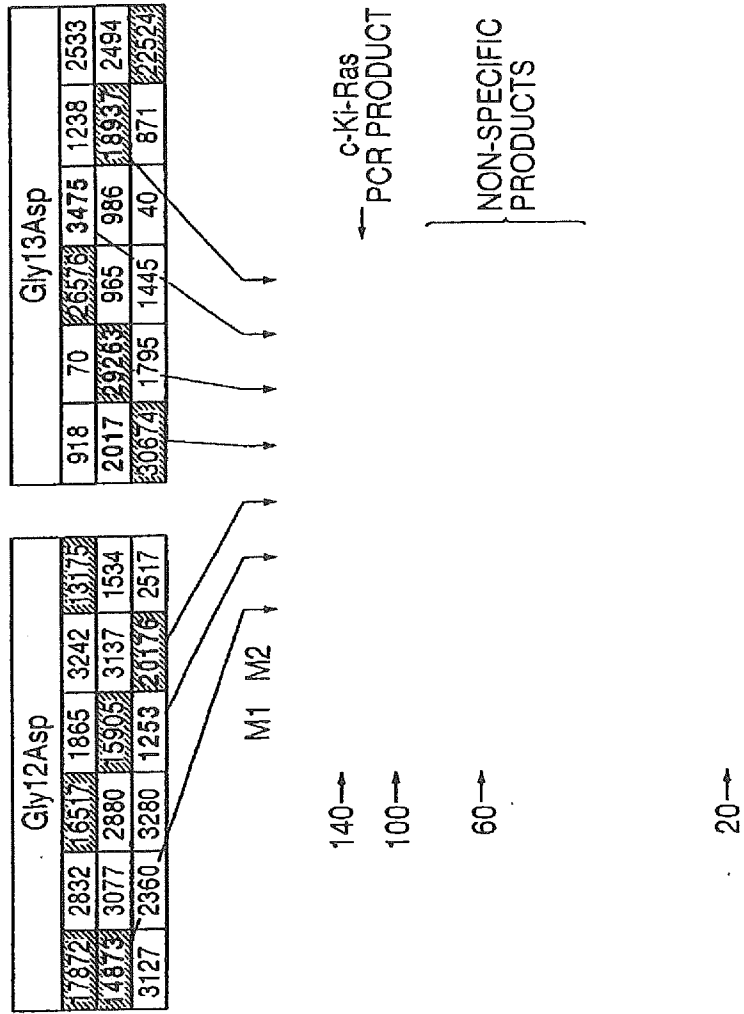


FIG. 2



**FIG. 3**



**FIG. 4**

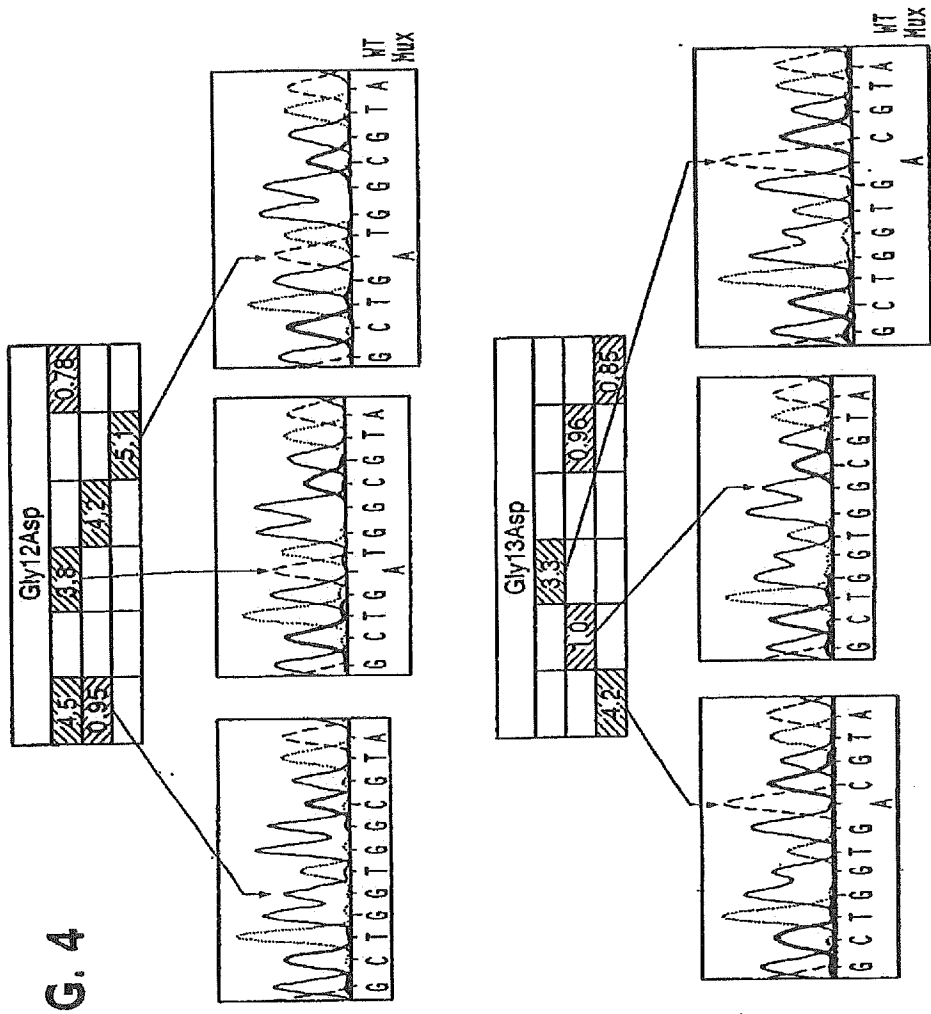
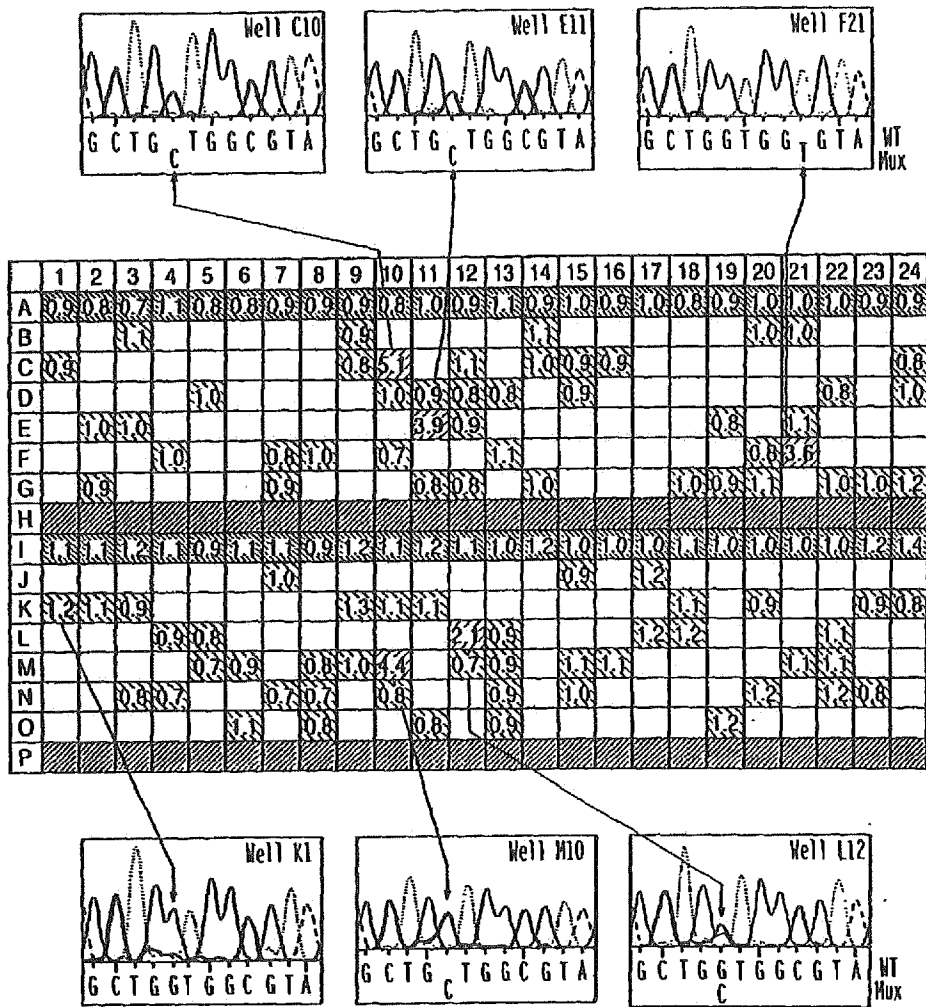


FIG. 5



PATENT  DESIGN B&W Ref. 01107-0001 Date May 21, 2002

HAND CARRY Group/Section Bldg Rm

Serial/Patent No. 091613, 826 Atty/Sec SAK/MHS/SEL

Inventor Bert Vogelstein et al Client JHU

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Application:  CIP  Continuation  Divisional

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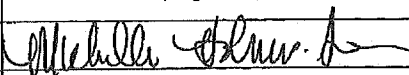


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|                                                                                            | Filing Date            | July 11, 2000   |
|                                                                                            | First Named Inventor   | Bert Vogelstein |
|                                                                                            | Group Art Unit         | 1637            |
|                                                                                            | Examiner Name          | Jeffrey Slew    |
| Total Number of Pages In This Submission                                                   | Attorney Docket Number | 001107.00031    |

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Bert Vogelstein, et. al. ) Group Art Unit: 1637  
Serial No. 09/613,826 ) Examiner: Jeffrey Siew  
Filing Date: July 11, 2000 ) Docket No. 01107.00031  
For: DIGITAL AMPLIFICATION

SUBMISSION OF FORMAL DRAWINGS

Assistant Commissioner of Patents  
Washington, D.C. 20231  
Attn: Box ISSUE FEE

Dear Sir:

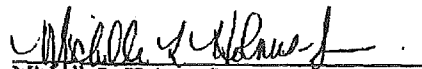
Attached hereto for filing in the United States Patent and Trademark Office are seven (7) sheets of formal drawings (FIGS. 1 A-C, 2-5) for the above-identified patent application.

Please charge any fee in connection with the filing of these drawings to our Deposit Account No. 19-0733 and forward the recorded document to the undersigned. A duplicate of this sheet is enclosed.

Respectfully submitted,

Date: May 21, 2002

By:

  
\_\_\_\_\_  
Mitchell L. Holmes-Son  
Registration No. 47,660

Banner & Witcoff, Ltd.  
1001 G Street, N.W., Eleventh Floor  
Washington, D.C. 20001-4597  
(202) 508-9100

## File History Report

Paper number \_\_\_\_\_ is missing from the United States Patent Trademark Office's copy of the file History. No additional information is available.

The following page(s) \_\_\_\_\_ of paper number \_\_\_\_\_ is/are missing from the United States Patent and Trademark Office's original copy of the file history. No additional information is available

The following checked item(s) below of paper number \_\_\_\_\_ is/are missing from the United States Patent and Trademark Office's original copy of the file history. No additional information is available

- PTO 1449
- PTO 892
- PTO 948
- PTO 1474
- Assignment
- Cover page

**Additional comments:**Paper #25 same as Paper #23



UNITED STATES PATENT AND TRADEMARK OFFICE

COMMISSIONER FOR PATENTS  
UNITED STATES PATENT AND TRADEMARK OFFICE  
WASHINGTON, D.C. 20231  
www.uspto.gov

# Fax Cover Sheet

Date: 15 Apr 2002

|                                                                                                                                                                                       |                              |
|---------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------|------------------------------|
| To: Michele L Holmes-Son                                                                                                                                                              | From: Jeffrey Siew           |
| Application/Control Number: 09/613,826                                                                                                                                                | Art Unit: 1656               |
| Fax No.: (202) 508-9299                                                                                                                                                               | Phone No.: 703-305-3886      |
| Voice No.: (202) 508-8100                                                                                                                                                             | Return Fax No.: 703-308-4556 |
| Re:                                                                                                                                                                                   | CC:                          |
| <input type="checkbox"/> Urgent <input type="checkbox"/> For Review <input type="checkbox"/> For Comment <input type="checkbox"/> For Reply <input type="checkbox"/> Per Your Request |                              |

Comments:  
per your request

Number of pages 4 including this page

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Assistant Commissioner for Patents  
Washington, DC 20231

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001

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# Fax Cover Sheet

Date: 28 Jun 2001

|                                        |                              |
|----------------------------------------|------------------------------|
| To: Ms. Holmeson                       | From: Jeffrey Slew           |
| Application/Control Number: 09/813,828 | Art Unit: 1636               |
| Fax No.: (202) 508-9299                | Phone No.: 703-305-3888      |
| Voice No.: (202) 508-9100              | Return Fax No.: 703-308-4556 |
| Re:                                    | CC:                          |

Urgent    For Review    For Comment    For Reply    For Your Request

Comments:

Per your request a copy of the notice to comply for office action 4/12/01

thanks

Jeffrey Slew



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# Fax Cover Sheet

Date: 29 Jun 2001

|                                                                                                                                                                                                  |                              |
|--------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------|------------------------------|
| To: Ms. Holmson                                                                                                                                                                                  | From: Jeffrey Siew           |
| Application/Control Number: 09/613,826                                                                                                                                                           | Art Unit: 1656               |
| Fax No.: (202) 508-9299                                                                                                                                                                          | Phone No.: 703-305-3886      |
| Voice No.: (202) 508-9100                                                                                                                                                                        | Return Fax No.: 703-308-4556 |
| Re:                                                                                                                                                                                              | CC:                          |
| <input checked="" type="checkbox"/> Urgent <input type="checkbox"/> For Review <input type="checkbox"/> For Comment <input type="checkbox"/> For Reply <input type="checkbox"/> Per Your Request |                              |

**Comments:**

Per your request a copy of the notice to comply for office action 4/12/01

thanks

Jeffrey Siew

Number of pages 2 including this page

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Assistant Commissioner for Patents

Application No.: 09/613826

**NOTICE TO COMPLY WITH REQUIREMENTS FOR PATENT APPLICATIONS CONTAINING NUCLEOTIDE SEQUENCE AND/OR AMINO ACID SEQUENCE DISCLOSURES**

Applicant must file the items indicated below within the time period set the Office action to which the Notice is attached to avoid abandonment under 35 U.S.C. § 133 (extensions of time may be obtained under the provisions of 37 CFR 1.136(a)).

The nucleotide and/or amino acid sequence disclosure contained in this application does not comply with the requirements for such a disclosure as set forth in 37 C.F.R. 1.821 - 1.825 for the following reason(s):

- 1. This application clearly fails to comply with the requirements of 37 C.F.R. 1.821-1.825. Applicant's attention is directed to the final rulemaking notice published at 55 FR 18230 (May 1, 1990), and 1114 OG 29 (May 15, 1990). If the effective filing date is on or after July 1, 1998, see the final rulemaking notice published at 63 FR 29620 (June 1, 1998) and 1211 OG 82 (June 23, 1998).
- 2. This application does not contain, as a separate part of the disclosure on paper copy, a "Sequence Listing" as required by 37 C.F.R. 1.821(c).
- 3. A copy of the "Sequence Listing" in computer readable form has not been submitted as required by 37 C.F.R. 1.821(e).
- 4. A copy of the "Sequence Listing" in computer readable form has been submitted. However, the content of the computer readable form does not comply with the requirements of 37 C.F.R. 1.822 and/or 1.823, as indicated on the attached copy of the marked-up "Raw Sequence Listing."
- 5. The computer readable form that has been filed with this application has been found to be damaged and/or unreadable as indicated on the attached CRF Diskette Problem Report. A Substitute computer readable form must be submitted as required by 37 C.F.R. 1.825(d).
- 6. The paper copy of the "Sequence Listing" is not the same as the computer readable form of the "Sequence Listing" as required by 37 C.F.R. 1.821(e).
- 7. Other: \_\_\_\_\_

**Applicant Must Provide:**

- An initial or substitute computer readable form (CRF) copy of the "Sequence Listing".
- An initial or substitute paper copy of the "Sequence Listing", as well as an amendment directing its entry into the specification.
- A statement that the content of the paper and computer readable copies are the same and, where applicable, include no new matter, as required by 37 C.F.R. 1.821(e) or 1.821(f) or 1.821(g) or 1.825(b) or 1.825(d).

For questions regarding compliance to these requirements, please contact:

For Rules Interpretation, call (703) 308-4216

For CRF Submission Help, call (703) 308-4212

PatentIn Software Program Support

Technical Assistance.....703-287-0200

To Purchase PatentIn Software.....703-306-2600

**PLEASE RETURN A COPY OF THIS NOTICE WITH YOUR REPLY**

THIS Form is for INTERNAL PTO USE ONLY  
It does NOT get mailed to the applicant.

NOTICE OF FILING / CLAIM FEE(S) DUE  
(CALCULATION SHEET)

APPLICATION NUMBER: 041613826

Total Fee Calculation

| Fee Code                     | Total # Claims | Number Exam | X  | Fee | Fee | Total       |
|------------------------------|----------------|-------------|----|-----|-----|-------------|
| Basic Filing Fee             | 011001         |             |    |     | 650 |             |
| Total Claims > 25            | 012001         | 64          | 25 | 44  | 792 |             |
| Independent Claims > 1       | 012101         | 5           | 1  | 2   | 104 |             |
| Multi-Dep. Claim Process     | 012104         |             |    |     | 130 |             |
| Surcharge                    | 011111         |             |    |     |     |             |
| English Translation          | 111            |             |    |     |     |             |
| <b>TOTAL FEE CALCULATION</b> |                |             |    |     |     | <b>1768</b> |

Fees due upon filing the application:

Total Filing Fees Due = \$ 1768  
 Less Filing Fees Submitted = \$ —  
**BALANCE DUE = \$ 1768**

[Signature]  
 Office of Initial Patent Examination

Figure 7




PATENT COOPERATION TREATY

From the INTERNATIONAL SEARCHING AUTHORITY 01107.00030

PCT

|                                                                                                                                                                                                                                                                        |                                                                                                                                                                                                                                                                                                                                                                                        |
|------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------|----------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------|
| <p>To:<br/> <b>BANNER &amp; WITCOFF, LTD.</b><br/>                 Attn. KAGAN, Sarah A.<br/>                 1001 G Street, N.W.<br/>                 Eleventh Floor<br/>                 Washington, DC 20001-4597<br/>                 UNITED STATES OF AMERICA</p> | <p style="text-align: center;"><b>DOCKETED</b></p> <p style="text-align: center;">JAN 2<br/>                 Central 19 Amendment<br/>                 21 FEB 2002</p> <p style="text-align: right;">NOTIFICATION OF TRANSMITTAL OF<br/>                 THE INTERNATIONAL SEARCH REPORT<br/>                 OR THE DECLARATION</p> <p style="text-align: right;">(PCT Rule 44.1)</p> |
| <p>Date of mailing<br/>                 (day/month/year)</p>                                                                                                                                                                                                           | <p style="text-align: right;">21/12/2001</p>                                                                                                                                                                                                                                                                                                                                           |
| <p>Applicant's or agent's file reference</p>                                                                                                                                                                                                                           | <p><b>01107.00030</b></p>                                                                                                                                                                                                                                                                                                                                                              |
| <p>FOR FURTHER ACTION</p>                                                                                                                                                                                                                                              | <p>See paragraphs 1 and 4 below</p>                                                                                                                                                                                                                                                                                                                                                    |
| <p>International application No.</p>                                                                                                                                                                                                                                   | <p><b>PCT/US 00/20740</b></p>                                                                                                                                                                                                                                                                                                                                                          |
| <p>International filing date<br/>                 (day/month/year)</p>                                                                                                                                                                                                 | <p style="text-align: right;"><b>31/07/2000</b></p>                                                                                                                                                                                                                                                                                                                                    |
| <p>Applicant</p>                                                                                                                                                                                                                                                       |                                                                                                                                                                                                                                                                                                                                                                                        |
| <p><b>THE JOHNS HOPKINS UNIVERSITY et al.</b></p>                                                                                                                                                                                                                      |                                                                                                                                                                                                                                                                                                                                                                                        |

1.  The applicant is hereby notified that the International Search Report has been established and is transmitted herewith.  
 Filing of amendments and statement under Article 19:  
 The applicant is entitled, if he so wishes, to amend the claims of the International Application (see Rule 46):  
  
 When? The time limit for filing such amendments is normally 2 months from the date of transmittal of the International Search Report; however, for more details, see the notes on the accompanying sheet.  
  
 Where? Directly to the International Bureau of WIPO  
 34, chemin des Colombettes  
 1211 Geneva 20, Switzerland  
 Facsimile No.: (41-22) 740.14.35  
  
 For more detailed instructions, see the notes on the accompanying sheet.
2.  The applicant is hereby notified that no International Search Report will be established and that the declaration under Article 17(2)(a) to that effect is transmitted herewith.
3.  With regard to the protest against payment of (an) additional fee(s) under Rule 40.2, the applicant is notified that:
  - the protest together with the decision thereon has been transmitted to the International Bureau together with the applicant's request to forward the texts of both the protest and the decision thereon to the designated Offices.
  - no decision has been made yet on the protest; the applicant will be notified as soon as a decision is made.
4. Further action(s): The applicant is reminded of the following:  
  
 Shortly after 18 months from the priority date, the international application will be published by the International Bureau. If the applicant wishes to avoid or postpone publication, a notice of withdrawal of the international application, or of the priority claim, must reach the International Bureau as provided in Rules 80bis.1 and 80bis.3, respectively, before the completion of the technical preparations for international publication.  
  
 Within 18 months from the priority date, a demand for international preliminary examination must be filed if the applicant wishes to postpone the entry into the national phase until 30 months from the priority date (in some Offices even later).  
  
 Within 20 months from the priority date, the applicant must perform the prescribed acts for entry into the national phase before all designated Offices which have not been elected in the demand or in a later election within 19 months from the priority date or could not be elected because they are not bound by Chapter II.

|                                                                                                                                                                                                                                                                                                                                                                        |                                                                                       |
|------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------|---------------------------------------------------------------------------------------|
| <p>Name and mailing address of the International Searching Authority</p> <p> European Patent Office, P.B. 5818 Patentaan 2<br/>                 NL-2280 HV Rijswijk<br/>                 Tel. (+31-70) 340-2040, Tx. 31 651 epo nl,<br/>                 Fax: (+31-70) 340-3016</p> | <p>Authorized officer</p> <p style="text-align: center;"><b>Catherine Humbert</b></p> |
|------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------|---------------------------------------------------------------------------------------|

## NOTES TO FORM PCT/ISA/220

These Notes are intended to give the basic instructions concerning the filing of amendments under article 19. The Notes are based on the requirements of the Patent Cooperation Treaty, the Regulations and the Administrative Instructions under that Treaty. In case of discrepancy between these Notes and those requirements, the latter are applicable. For more detailed information, see also the PCT Applicant's Guide, a publication of WIPO.

In these Notes, "Article", "Rule", and "Section" refer to the provisions of the PCT, the PCT Regulations and the PCT Administrative Instructions respectively.

### INSTRUCTIONS CONCERNING AMENDMENTS UNDER ARTICLE 19

The applicant has, after having received the international search report, one opportunity to amend the claims of the international application. It should however be emphasized that, since all parts of the international application (claims, description and drawings) may be amended during the international preliminary examination procedure, there is usually no need to file amendments of the claims under Article 19 except where, e.g. the applicant wants the letter to be published for the purposes of provisional protection or has another reason for amending the claims before international publication. Furthermore, it should be emphasized that provisional protection is available in some States only.

#### What parts of the international application may be amended?

Under Article 19, only the claims may be amended.

During the international phase, the claims may also be amended (or further amended) under Article 34 before the International Preliminary Examining Authority. The description and drawings may only be amended under Article 34 before the International Examining Authority.

Upon entry into the national phase, all parts of the international application may be amended under Article 28 or, where applicable, Article 41.

#### When?

Within 2 months from the date of transmittal of the international search report or 16 months from the priority date, whichever time limit expires later. It should be noted, however, that the amendments will be considered as having been received on time if they are received by the International Bureau after the expiration of the applicable time limit but before the completion of the technical preparations for international publication (Rule 46.1).

#### Where not to file the amendments?

The amendments may only be filed with the International Bureau and not with the receiving Office or the International Searching Authority (Rule 46.2).

Where a demand for international preliminary examination has been/is filed, see below.

#### How?

Either by cancelling one or more entire claims, by adding one or more new claims or by amending the text of one or more of the claims as filed.

A replacement sheet must be submitted for each sheet of the claims which, on account of an amendment or amendments, differs from the sheet originally filed.

All the claims appearing on a replacement sheet must be numbered in Arabic numerals. Where a claim is canceled, no renumbering of the other claims is required. In all cases where claims are renumbered, they must be renumbered consecutively (Administrative Instructions, Section 205(b)).

The amendments must be made in the language in which the international application is to be published.

#### What documents must/may accompany the amendments?

Letter (Section 265(b)):

The amendments must be submitted with a letter.

The letter will not be published with the international application and the amended claims. It should not be confused with the "Statement under Article 19(1)" (see below, under "Statement under Article 19(1)").

The letter must be in English or French, at the choice of the applicant. However, if the language of the international application is English, the letter must be in English; if the language of the international application is French, the letter must be in French.

## NOTES TO FORM PCT/ISA/220 (continued)

The letter must indicate the differences between the claims as filed and the claims as amended. It must, in particular, indicate, in connection with each claim appearing in the international application (it being understood that identical indications concerning several claims may be grouped), whether

- (i) the claim is unchanged;
- (ii) the claim is cancelled;
- (iii) the claim is new;
- (iv) the claim replaces one or more claims as filed;
- (v) the claim is the result of the division of a claim as filed.

The following examples illustrate the manner in which amendments must be explained in the accompanying letter:

1. [Where originally there were 48 claims and after amendment of some claims there are 51]:  
"Claims 1 to 29, 31, 32, 34, 35, 37 to 48 replaced by amended claims bearing the same numbers; claims 30, 33 and 36 unchanged; new claims 49 to 51 added."
2. [Where originally there were 15 claims and after amendment of all claims there are 11]:  
"Claims 1 to 15 replaced by amended claims 1 to 11."
3. [Where originally there were 14 claims and the amendments consist in cancelling some claims and in adding new claims]:  
"Claims 1 to 6 and 14 unchanged; claims 7 to 13 cancelled; new claims 15, 16 and 17 added." or  
"Claims 7 to 13 cancelled; new claims 15, 16 and 17 added; all other claims unchanged."
4. [Where various kinds of amendments are made]:  
"Claims 1-10 unchanged; claims 11 to 13, 18 and 19 cancelled; claims 14, 15 and 16 replaced by amended claim 14; claim 17 subdivided into amended claims 15, 16 and 17; new claims 20 and 21 added."

### "Statement under article 19(1)" (Rule 49.4)

The amendments may be accompanied by a statement explaining the amendments and indicating any impact that such amendments might have on the description and the drawings (which cannot be amended under Article 19(1)).

The statement will be published with the international application and the amended claims.

It must be in the language in which the international application is to be published.

It must be brief, not exceeding 500 words if in English or if translated into English.

It should not be confused with and does not replace the letter indicating the differences between the claims as filed and as amended. It must be filed on a separate sheet and must be identified as such by a heading, preferably by using the words "Statement under Article 19(1)."

It may not contain any disparaging comments on the international search report or the relevance of citations contained in that report. Reference to citations, relevant to a given claim, contained in the international search report may be made only in connection with an amendment of that claim.

### Consequence if a demand for international preliminary examination has already been filed

If, at the time of filing any amendments under Article 19, a demand for international preliminary examination has already been submitted, the applicant must preferably, at the same time of filing the amendments with the International Bureau, also file a copy of such amendments with the International Preliminary Examining Authority (see Rule 62.2(a), first sentence).

### Consequences with regard to translation of the international application for entry into the national phase

The applicant's attention is drawn to the fact that, where upon entry into the national phase, a translation of the claims as amended under Article 19 may have to be furnished to the designated/elected Offices, instead of, or in addition to, the translation of the claims as filed.

For further details on the requirements of each designated/elected Office, see Volume II of the PCT Applicant's Guide.

PATENT COOPERATION TREATY

PCT

INTERNATIONAL SEARCH REPORT

(PCT Article 18 and Rules 43 and 44)

|                                                             |                                                                                                                                                         |                                                                |
|-------------------------------------------------------------|---------------------------------------------------------------------------------------------------------------------------------------------------------|----------------------------------------------------------------|
| Applicant's or agent's file reference<br><b>01107.00030</b> | <b>FOR FURTHER ACTION</b> see Notification of Transmittal of International Search Report (Form PCT/ISA/220) as well as, where applicable, Item 5 below. |                                                                |
| International application No.<br><b>PCT/US 00/20740</b>     | International filing date (day/month/year)<br><b>31/07/2000</b>                                                                                         | (Earliest) Priority Date (day/month/year)<br><b>02/08/1999</b> |
| Applicant<br><b>THE JOHNS HOPKINS UNIVERSITY et al.</b>     |                                                                                                                                                         |                                                                |

This International Search Report has been prepared by this International Searching Authority and is transmitted to the applicant according to Article 18. A copy is being transmitted to the International Bureau.

This International Search Report consists of a total of 5 sheets.  
 It is also accompanied by a copy of each prior art document cited in this report.

1. Basis of the report

- a. With regard to the language, the International search was carried out on the basis of the International application in the language in which it was filed, unless otherwise indicated under this item.
- the International search was carried out on the basis of a translation of the International application furnished to this Authority (Rule 23.1(b)).
- b. With regard to any nucleotide and/or amino acid sequence disclosed in the International application, the International search was carried out on the basis of the sequence listing:
- contained in the International application in written form.
- filed together with the International application in computer readable form.
- furnished subsequently to this Authority in written form.
- furnished subsequently to this Authority in computer readable form.
- the statement that the subsequently furnished written sequence listing does not go beyond the disclosure in the International application as filed has been furnished.
- the statement that the information recorded in computer readable form is identical to the written sequence listing has been furnished.

2.  Certain claims were found unsearchable (See Box I).
3.  Unity of invention is lacking (see Box I).

4. With regard to the title,

- the text is approved as submitted by the applicant.
- the text has been established by this Authority to read as follows:

5. With regard to the abstract,

- the text is approved as submitted by the applicant.
- the text has been established, according to Rule 38.2(b), by this Authority as it appears in Box III. The applicant may, within one month from the date of mailing of this International search report, submit comments to this Authority.

6. The figure of the drawings to be published with the abstract is Figure No.

- as suggested by the applicant.
- because the applicant failed to suggest a figure.
- because this figure better characterizes the invention.
- None of the figures.

Form PCT/ISA/210 (first sheet) (July 1998)

**INTERNATIONAL SEARCH REPORT**

International Application No  
PCT/US 00/20740

|                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                            |                                                                                                                                                                                                                                                                                                                                     |                                                                              |
|------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------|-------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------|------------------------------------------------------------------------------|
| <b>A. CLASSIFICATION OF SUBJECT MATTER</b><br>IPC 7 C12Q1/68                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                               |                                                                                                                                                                                                                                                                                                                                     |                                                                              |
| According to International Patent Classification (IPC) or to both national classification and IPC                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                          |                                                                                                                                                                                                                                                                                                                                     |                                                                              |
| <b>B. FIELDS SEARCHED</b>                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                  |                                                                                                                                                                                                                                                                                                                                     |                                                                              |
| Minimum documentation searched (classification system followed by classification symbols)<br>IPC 7 C12Q                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                    |                                                                                                                                                                                                                                                                                                                                     |                                                                              |
| Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                              |                                                                                                                                                                                                                                                                                                                                     |                                                                              |
| Electronic data base consulted during the International search (name of data base and, where practical, search terms used)<br>EPO-Internal, WPI Data, PAJ, BIOSIS, CHEM ABS Data, EMBASE, MEDLINE, SCISEARCH                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                               |                                                                                                                                                                                                                                                                                                                                     |                                                                              |
| <b>C. DOCUMENTS CONSIDERED TO BE RELEVANT</b>                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                              |                                                                                                                                                                                                                                                                                                                                     |                                                                              |
| Category *                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                 | Citation of document, with indication, where appropriate, of the relevant passages                                                                                                                                                                                                                                                  | Relevant to claim No.                                                        |
| X                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                          | VET JACQUELINE A M ET AL: "Multiplex detection of four pathogenic retroviruses using molecular beacons."<br>PROCEEDINGS OF THE NATIONAL ACADEMY OF SCIENCES OF THE UNITED STATES, vol. 96, no. 11, 25 May 1999 (1999-05-25), pages 6394-6399, XP002145609<br>May 25, 1999<br>ISSN: 0027-8424<br>the whole document<br>-----<br>-/-- | 1-13,<br>15-19,<br>21-24,<br>30, 32,<br>38-45,<br>47-51,<br>53-56,<br>62, 64 |
| <input checked="" type="checkbox"/> Further documents are listed in the continuation of box C. <input checked="" type="checkbox"/> Patent family members are listed in annex.                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                              |                                                                                                                                                                                                                                                                                                                                     |                                                                              |
| * Special categories of cited documents:                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                   |                                                                                                                                                                                                                                                                                                                                     |                                                                              |
| *A* document defining the general state of the art which is not considered to be of particular relevance<br>*E* earlier document but published on or after the International filing date<br>*L* document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)<br>*O* document referring to an oral disclosure, use, exhibition or other means<br>*P* document published prior to the international filing date but later than the priority date claimed<br>*T* later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention<br>*X* document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone<br>*Y* document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.<br>*Z* document member of the same patent family |                                                                                                                                                                                                                                                                                                                                     |                                                                              |
| Date of the actual completion of the international search                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                  | Date of making of the international search report                                                                                                                                                                                                                                                                                   |                                                                              |
| 10 December 2001                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                           | 21/12/2001                                                                                                                                                                                                                                                                                                                          |                                                                              |
| Name and mailing address of the ISA<br>European Patent Office, P.B. 5818 Patentlaan 2<br>NL - 2280 HV Rijswijk<br>Tel. (+31-70) 340-2040, Tx 31 651 epo nl,<br>Fax: (+31-70) 340-3016                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                      | Authorized officer<br><br>Gabriels, J                                                                                                                                                                                                                                                                                               |                                                                              |

## INTERNATIONAL SEARCH REPORT

International Application No

PCT/US 00/20740

| C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT |                                                                                                                                                                                                                                                                           |                                                                                                                    |
|------------------------------------------------------|---------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------|--------------------------------------------------------------------------------------------------------------------|
| Category *                                           | Citation of document, with indication, where appropriate, of the relevant passages                                                                                                                                                                                        | Relevant to claim No.                                                                                              |
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Form PCT/ISA/210 (continuation of second sheet) (July 1992)

page 2 of 3

INTERNATIONAL SEARCH REPORT

International Application No  
PCT/US 00/20740

| C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT |                                                                                                                                                                                                                                                                                                                                                                                                                                  |                       |
|------------------------------------------------------|----------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------|-----------------------|
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**INTERNATIONAL SEARCH REPORT**

Information on patent family members

International Application No

PCT/US 00/20740

| Patent document cited in search report | Publication date | Patent family member(s) | Publication date |
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|                                                                                   |             |                                                                                                                                 |                                                |                                    |            |                                                                       |        |                |
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| <b>PATENT APPLICATION FEE DETERMINATION RECORD</b><br>Effective December 29, 1999 |             |                                                                                                                                 |                                                | Application or Docket Number       |            |                                                                       |        |                |
| <b>CLAIMS AS FILED - PART I</b>                                                   |             |                                                                                                                                 |                                                |                                    |            | SMALL ENTITY TYPE <input type="checkbox"/> OR OTHER THAN SMALL ENTITY |        |                |
|                                                                                   |             | (Column 1)                                                                                                                      |                                                |                                    | (Column 2) |                                                                       |        |                |
| FOR                                                                               |             | NUMBER FILED                                                                                                                    |                                                | NUMBER EXTRA                       |            | RATE                                                                  | FEE    |                |
| BASIC FEE                                                                         |             |                                                                                                                                 |                                                |                                    |            |                                                                       | 345.00 |                |
| TOTAL CLAIMS                                                                      |             | 44                                                                                                                              | minus 20=                                      |                                    | 44         | X\$ 9=                                                                |        |                |
| INDEPENDENT CLAIMS                                                                |             | 5                                                                                                                               | minus 3=                                       |                                    | 2          | X39=                                                                  |        |                |
|                                                                                   |             | MULTIPLE DEPENDENT CLAIM PRESENT                                                                                                |                                                |                                    |            |                                                                       |        |                |
|                                                                                   |             | * If the difference in column 1 is less than zero, enter "0" in column 2                                                        |                                                |                                    |            |                                                                       |        |                |
| <b>CLAIMS AS AMENDED - PART II</b>                                                |             |                                                                                                                                 |                                                |                                    |            | SMALL ENTITY OR OTHER THAN SMALL ENTITY                               |        |                |
|                                                                                   |             | (Column 1)                                                                                                                      |                                                |                                    | (Column 2) | (Column 3)                                                            |        |                |
| AMENDMENT A                                                                       |             | CLAIMS REMAINING AFTER AMENDMENT                                                                                                |                                                | HIGHEST NUMBER PREVIOUSLY PAID FOR |            | PRESENT EXTRA                                                         | RATE   | ADDITIONAL FEE |
|                                                                                   | Total       | *                                                                                                                               | Minus                                          | **                                 |            | =                                                                     | X\$ 9= |                |
|                                                                                   | Independent | *                                                                                                                               | Minus                                          | ***                                |            | =                                                                     | X39=   |                |
|                                                                                   |             |                                                                                                                                 | FIRST PRESENTATION OF MULTIPLE DEPENDENT CLAIM |                                    |            |                                                                       |        |                |
|                                                                                   |             | * If the difference in column 1 is less than zero, enter "0" in column 2                                                        |                                                |                                    |            |                                                                       |        |                |
|                                                                                   |             | * 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. |                                                |                                    |            |                                                                       |        |                |

# EXHIBIT 4



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| APPLICATION NUMBER | PATENT NUMBER | GROUP ART UNIT | FILE WRAPPER LOCATION |
|--------------------|---------------|----------------|-----------------------|
| 60/146,792         |               |                | 9200I 0041 061 05     |



**Correspondence Address/Fee Address Change**

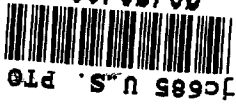
The following fields have been set to Customer Number 11332 on 10/24/2011

- 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

66/20/80



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### PROVISIONAL APPLICATION COVER SHEET

*Approv*

This is a request for filing a PROVISIONAL APPLICATION under 37 CFR 1.53(c)(2).

|               |             |                                        |   |
|---------------|-------------|----------------------------------------|---|
| Docket Number | 01107.81418 | Type a plus sign (+) inside this box - | + |
|---------------|-------------|----------------------------------------|---|

| INVENTOR(s)/APPLICANT(s)                                                                                                           |                 |                  |                                              |
|------------------------------------------------------------------------------------------------------------------------------------|-----------------|------------------|----------------------------------------------|
| LAST NAME                                                                                                                          | FIRST NAME      | M.I.             | RESIDENCE (CITY and either STATE or COUNTRY) |
| VOGELSTEIN<br>KINZLER                                                                                                              | Bert<br>Kenneth | W.               | Baltimore, Maryland<br>Belair, Maryland      |
| TITLE OF THE INVENTION (280 characters max)                                                                                        |                 |                  |                                              |
| DIGITAL AMPLIFICATION                                                                                                              |                 |                  |                                              |
| CORRESPONDENCE ADDRESS                                                                                                             |                 |                  |                                              |
| BANNER & WITCOFF, LTD.<br>Eleventh Floor<br>1001 G Street, N.W.                                                                    |                 |                  |                                              |
| STATE Washington, D.C.                                                                                                             | ZIP 20001-4597  | COUNTRY USA      |                                              |
| ENCLOSED APPLICATION PARTS (check all that apply)                                                                                  |                 |                  |                                              |
| <input checked="" type="checkbox"/>                                                                                                | Specification   | Number of Pages  | 28                                           |
| <input checked="" type="checkbox"/>                                                                                                | Drawings        | Number of Sheets | 8.00                                         |
|                                                                                                                                    |                 |                  | Small Entity Statement                       |
|                                                                                                                                    |                 |                  | Other (specify)                              |
|                                                                                                                                    |                 |                  | CLAIMS 64                                    |
| METHOD OF PAYMENT (check one)                                                                                                      |                 |                  |                                              |
| <input type="checkbox"/> A check or money order is enclosed to cover the Provisional filing fee                                    |                 |                  |                                              |
| <input checked="" type="checkbox"/> The Commissioner is hereby authorized to charge filing fees and credit Deposit Account Number. |                 | 19-0733          | PROVISIONAL FILING FEE AMOUNT (\$)           |



6688 U.S. PTO

The invention was made by an agency of the United States Government or under a contract with an agency of the United States Government.

NO  
 YES, the name of the U.S. Government agency and the Government contract number are: National Institute of Health CA 43460

Respectfully submitted, *Sarah A. Kagan*  
August 2, 1999

TYPED or PRINTED NAME Sarah A. Kagan REG. NO. (if appropriate) 32,141

Additional inventors are being named on separately numbered sheets attached hereto

### PROVISIONAL APPLICATION FILING ONLY

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## 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.

### **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.

5 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  
10 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 fluorescent 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 fluorescent 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.

30 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. 1. Schematic of experimental design. (A) 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. (B) 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.* . (C) 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 and Gly12Arg mutations, contaminating non-neoplastic cells within the tumor presumably accounted for the relatively low ratios. In the cases with Gly12Ser and Gly12Asp, there were apparently two or more alleles of mutant *c-Ki-Ras* for every WT allele; both these tumors were aneuploid.

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  $\sim 1.0$  contained WT sequences.

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  $1.0 \pm 0.1$  (mean  $\pm 1$  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.

## **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.

5 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.

10 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.

25 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 fluorescent 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 fluorescent probe would then be used to detect PCR products from the reference transcript and a second fluorescent 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.)

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.

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<sub>4</sub>SO<sub>4</sub>, 6.7 mM MgCl<sub>2</sub>, 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<sub>4</sub>SO<sub>4</sub>, 6.7 mM MgCl<sub>2</sub>, 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'; Primer R1:  
5'-TCTGAATTAGCTGTATCGTCAAGG-3'; Primer INT:  
5' - TAGCTGTATCGTCAAGGCAC - 3' ; MB - RED :  
5'-Cy3-CACGGGCCTGCTGAAAATGACTGCGTG-Dabcyl-3';

M B - G R E E N :  
5'-Fluorescein-CACGGGAGCTGGTGGCGTAGCGTG-Dabcyl-3'.  
Molecular Beacons were synthesized by Midland Scientific and other  
oligonucleotides were synthesized by Gene Link. All were dissolved at 50  $\mu$ M  
5 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  
10 5'-CATTATTTTATTATAAGGCCTGC-3'. 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  
20 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  
25 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  
30 energy resonance transfer is inversely proportional to the 6<sup>th</sup> 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  
5 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  
10 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  
15 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  
20 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  
25 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  
30

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 (T<sub>m</sub>) 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 T<sub>m</sub> 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 determining the ratio of a selected genetic sequence in a population of genetic sequences, 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 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 biological sample.

2. The method of claim 1 wherein the step of diluting is performed until at least one-tenth 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 for the step of analyzing to determine the presence of the selected genetic sequence.

3. 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.

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 10 nucleic acid template molecules containing the reference genetic sequence.

5. 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 the reference genetic sequence.

6. The method of claim 1 wherein the biological sample is cell-free.

7. The method of claim 1 wherein the number of assay samples within the set is greater than 10.

8. The method of claim 1 wherein the number of assay samples within the set is greater than 50.

9. The method of claim 1 wherein the number of assay samples within the set is greater than 100.

10. The method of claim 1 wherein the number of assay samples within the set is greater than 500.

11. The method of claim 1 wherein the number of assay samples within the set is greater than 1000.

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

13. 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 fluorescent dye at one of the 5' or 3' ends and a quenching agent at the opposite 5' or 3' end.



14. The method of claim 1 wherein the step of analyzing employs gel electrophoresis.

15. The method of claim 1 wherein the step of analyzing employs hybridization to at least one nucleic acid probe.

5 16. The method of claim 1 wherein the step of analyzing employs hybridization to at least two nucleic acid probe.

17. The method of claim 13 wherein two molecular beacon probes are used, each having a different fluorescent dye.

18. The method of claim 13 wherein the molecular beacon probe detects a wild-type selected genetic sequence better than a mutant selected genetic sequence.

19. The method of claim 1 wherein the step of amplifying employs a single pair of primers.

20. The method of claim 1 wherein the step of amplifying employs a polymerase which is activated only after heating.

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

22. The method of claim 1 wherein the step of amplifying employs at least 50 cycles of heating and cooling.

25 23. The method of claim 1 wherein the step of amplifying employs at least 60 cycles of heating and cooling.

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

25. 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.

26. The method of claim 1 wherein the selected genetic sequence is a translocated allele.

27. The method of claim 1 wherein the selected genetic sequence is a wild-type allele.

28. The method of claim 1 wherein the selected genetic sequence is within an amplicon which is amplified during neoplastic development.

29. The method of claim 1 wherein the selected genetic sequence is a rare exon sequence.

30. The method of claim 1 wherein the nucleic acid template molecules comprise cDNA of RNA transcripts and the selected genetic sequence is present on a cDNA of a first transcript and the reference genetic sequence is present on a cDNA of a second transcript.

31. The method of claim 1 wherein the selected genetic sequence comprises a first mutation and the reference genetic sequence comprises a second mutation.

32. The method of claim 1 wherein the selected genetic sequence and the reference genetic sequence are on distinct chromosomes.

33. A molecular beacon probe comprising:



38. A method for determining the ratio of a selected genetic sequence in a population of genetic sequences, 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;

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 biological sample.

39. The method of claim 38 wherein the number of assay samples within the set is greater than 10.

40. The method of claim 38 wherein the number of assay samples within the set is greater than 50.

41. The method of claim 38 wherein the number of assay samples within the set is greater than 100.

42. The method of claim 38 wherein the number of assay samples within the set is greater than 500.

43. The method of claim 38 wherein the number of assay samples within the set is greater than 1000.

44. The method of claim 38 wherein the step of amplifying and the step of analyzing are performed on assay samples in the same receptacle.

5

45. The method of claim 38 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 fluorescent dye at one of the 5' or 3' ends and a quenching agent at the opposite 5' or 3' end.

46. The method of claim 38 wherein the step of analyzing employs gel electrophoresis.

10

47. The method of claim 38 wherein the step of analyzing employs hybridization to at least one nucleic acid probe.

48. The method of claim 38 wherein the step of analyzing employs hybridization to at least two nucleic acid probe.

49. The method of claim 45 wherein two molecular beacon probes are used, each having a different fluorescent dye.

15

50. The method of claim 45 wherein the molecular beacon probe detects a wild-type selected genetic sequence better than a mutant selected genetic sequence.

20

51. The method of claim 38 wherein the step of amplifying employs a single pair of primers.

52. The method of claim 38 wherein the step of amplifying employs a polymerase which is activated only after heating.

25

53. The method of claim 38 wherein the step of amplifying employs at least 40 cycles of heating and cooling.

54. The method of claim 38 wherein the step of amplifying employs at least 50 cycles of heating and cooling.

55. The method of claim 38 wherein the step of amplifying employs at least 60 cycles of heating and cooling.

56. The method of claim 38 wherein the template molecules are obtained from a body sample selected from the group consisting of stool, blood, and lymph nodes.

57. The method of claim 38 wherein the template molecules are obtained from a body sample of a leukemia or lymphoma patient who has received anti-cancer therapy, said body sample being selected from the group consisting of blood and bone marrow.

58. The method of claim 38 wherein the selected genetic sequence is a translocated allele.

59. The method of claim 38 wherein the selected genetic sequence is a wild-type allele.

60. The method of claim 38 wherein the selected genetic sequence is within an amplicon which is amplified during neoplastic development.

61. The method of claim 38 wherein the selected genetic sequence is a rare exon sequence.

62. The method of claim 38 wherein the nucleic acid template molecules comprise cDNA of RNA transcripts and the selected genetic sequence is present on a cDNA of a first transcript and the reference genetic sequence is present on a cDNA of a second transcript.



## DIGITAL AMPLIFICATION

### ABSTRACT

5 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  
10 measure of the proportion of variant sequences within a DNA sample.



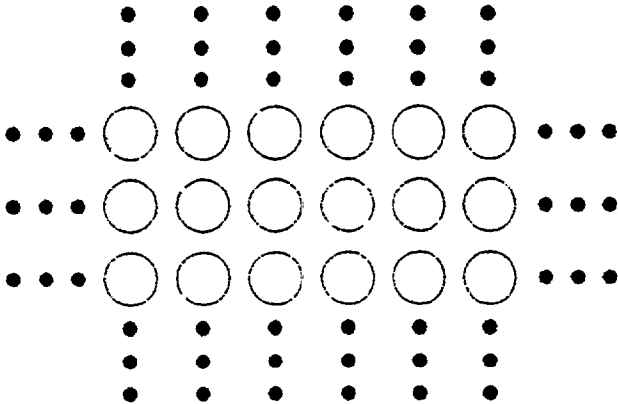
Fig. 1A

DNA

Step 1



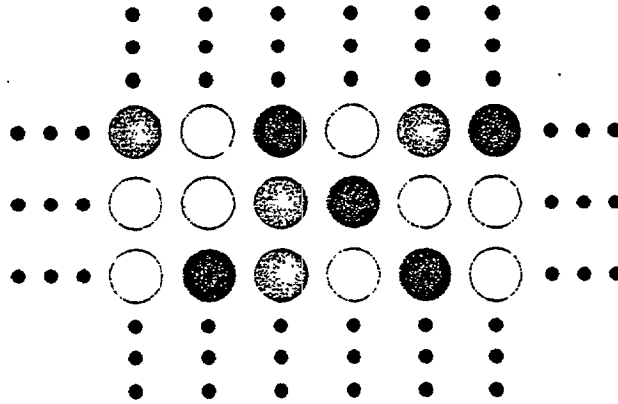
Dilute to ~1/2 copy/well  
PCR






Step 2



Add Fluorescent Probes  
Fluorometry



-  = No PCR Product
-  = Wild Type PCR Product
-  = Mutant PCR Product

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Fig. 1B

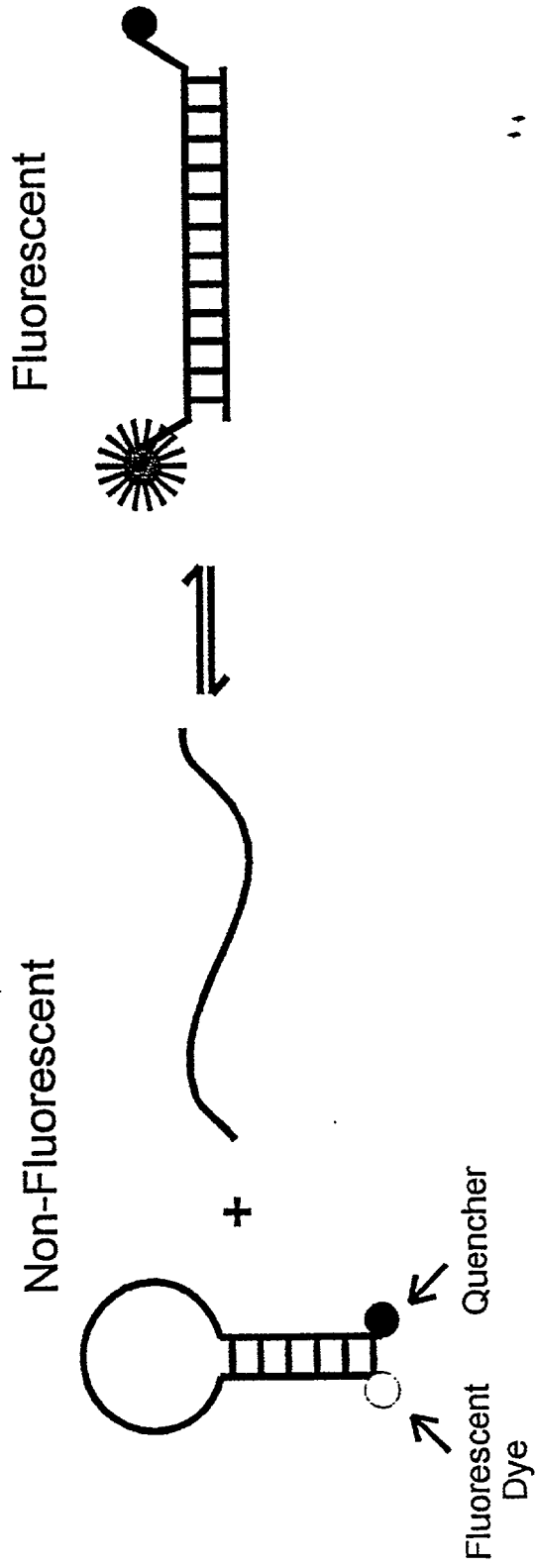
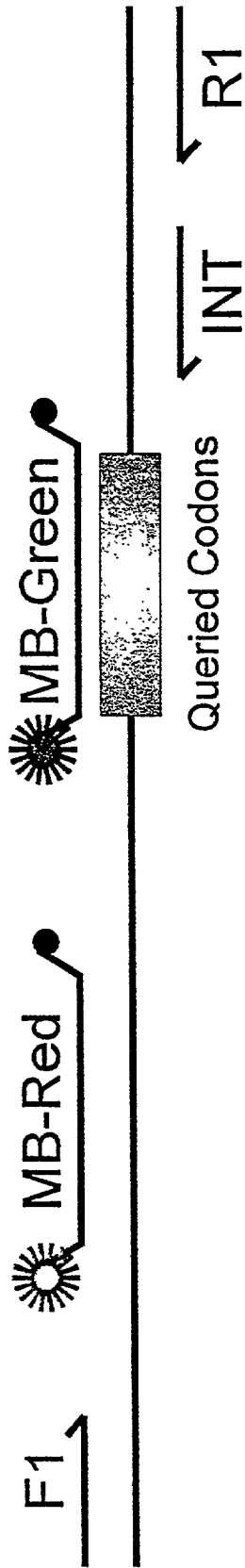
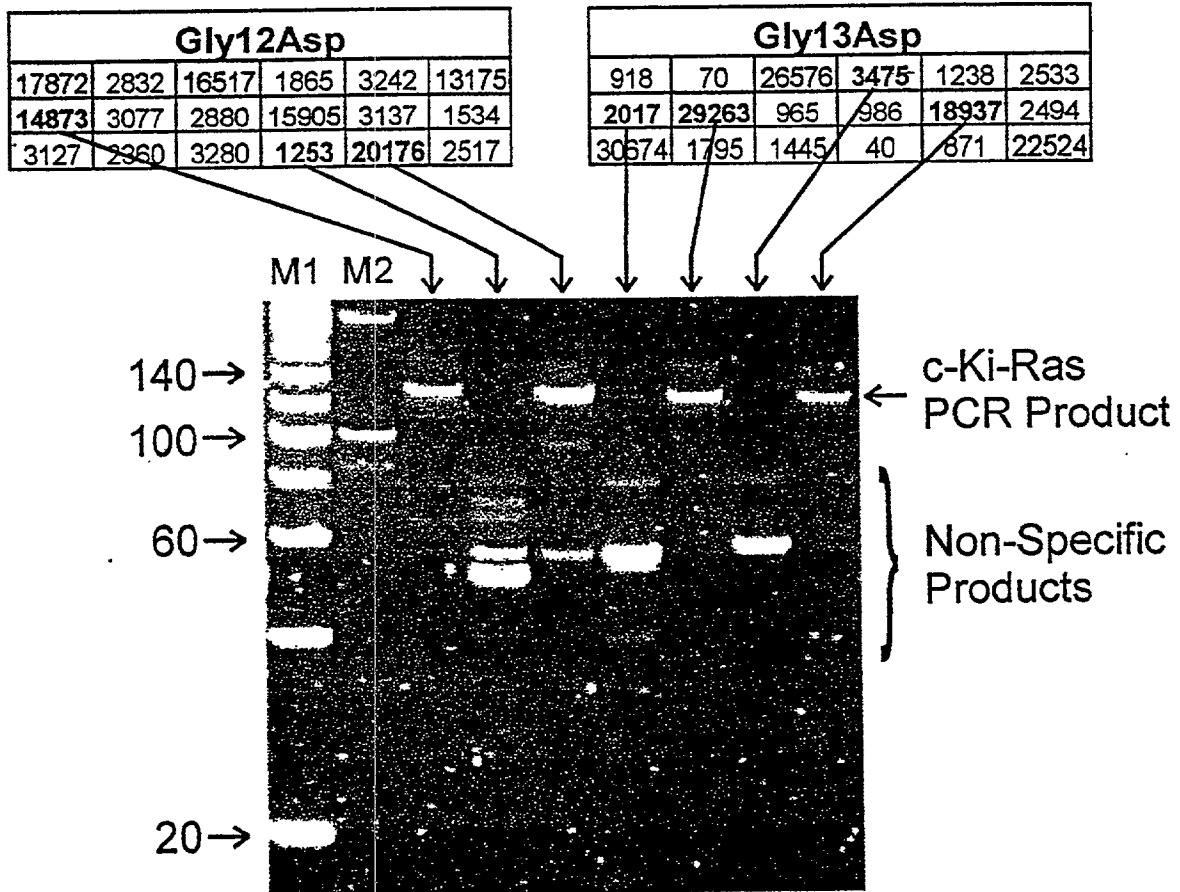


Fig. 1C





# Fig. 3



662080-2544409



# Fig. 5

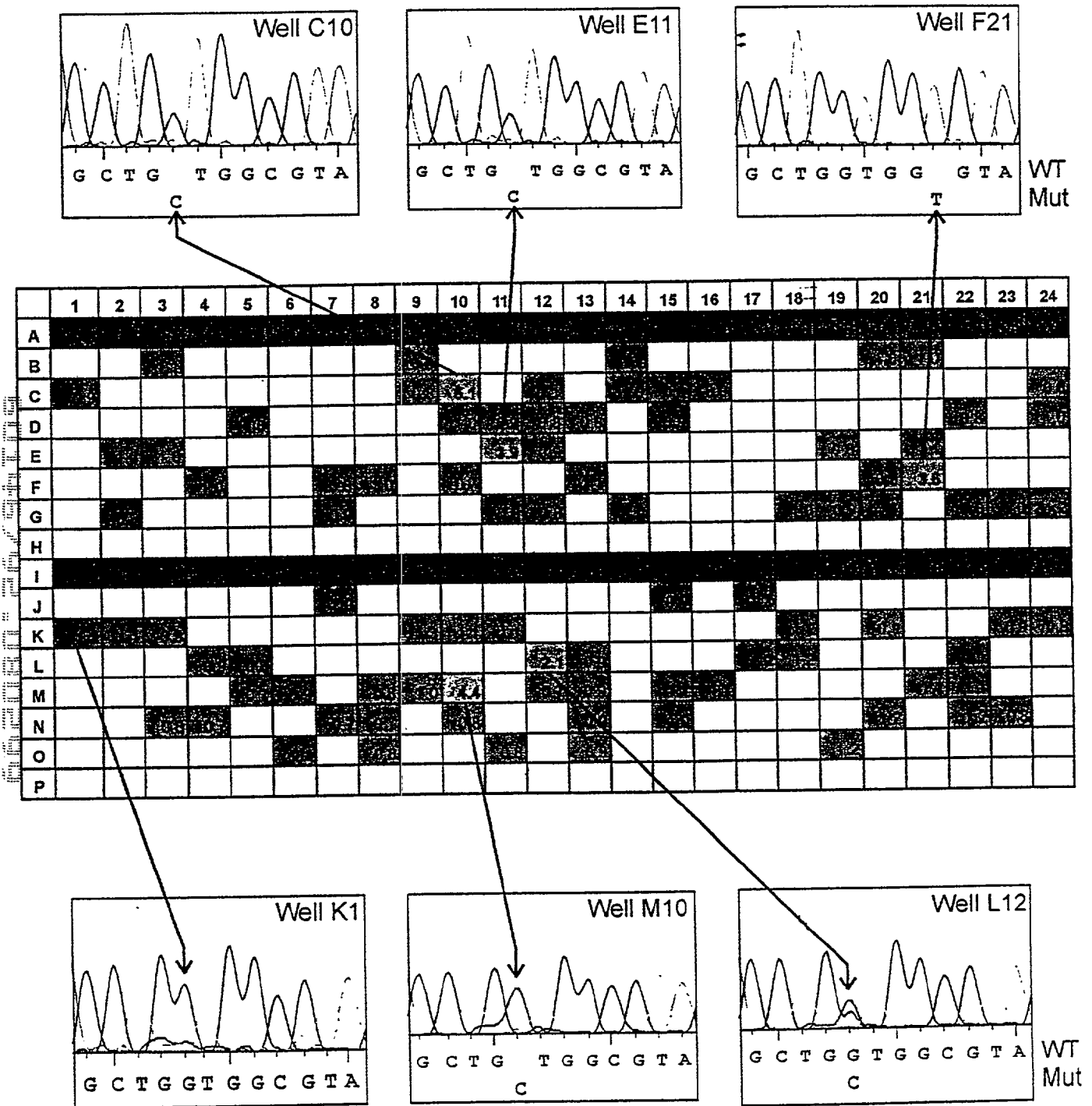


Fig. 6

**Table 1. Potential Applications of Dig-PCR**

| 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                    |



# EXHIBIT 5

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: <b>DIGITAL AMPLIFICATION</b> | ) | 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 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~~ 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

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.

| <b>PETITION FOR EXTENSION OF TIME UNDER 37 CFR 1.136(a)</b>                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                        | Docket Number (Optional)<br>001107.00866 |                         |                  |                         |  |                                                        |       |      |          |                                                                    |       |       |                  |                                                           |        |       |          |                                                          |        |       |          |                                                          |        |        |          |
|------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------|------------------------------------------|-------------------------|------------------|-------------------------|--|--------------------------------------------------------|-------|------|----------|--------------------------------------------------------------------|-------|-------|------------------|-----------------------------------------------------------|--------|-------|----------|----------------------------------------------------------|--------|-------|----------|----------------------------------------------------------|--------|--------|----------|
| Application Number 13/071,105                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                      | Filed March 24, 2011                     |                         |                  |                         |  |                                                        |       |      |          |                                                                    |       |       |                  |                                                           |        |       |          |                                                          |        |       |          |                                                          |        |        |          |
| For Digital Amplification                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                          |                                          |                         |                  |                         |  |                                                        |       |      |          |                                                                    |       |       |                  |                                                           |        |       |          |                                                          |        |       |          |                                                          |        |        |          |
| Art Unit 1637                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                      | Examiner Samuel C. Woolwine              |                         |                  |                         |  |                                                        |       |      |          |                                                                    |       |       |                  |                                                           |        |       |          |                                                          |        |       |          |                                                          |        |        |          |
| <p>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.</p> <p>The requested extension and fee are as follows (check time period desired and enter the appropriate fee below):</p> <table style="width: 100%; border-collapse: collapse;"> <thead> <tr> <th style="width: 40%;"></th> <th style="width: 15%; text-align: center;"><u>Fee</u></th> <th style="width: 15%; text-align: center;"><u>Small Entity Fee</u></th> <th style="width: 30%;"></th> </tr> </thead> <tbody> <tr> <td><input type="checkbox"/> One month (37 CFR 1.17(a)(1))</td> <td style="text-align: center;">\$150</td> <td style="text-align: center;">\$75</td> <td style="text-align: right;">\$ _____</td> </tr> <tr> <td><input checked="" type="checkbox"/> Two months (37 CFR 1.17(a)(2))</td> <td style="text-align: center;">\$560</td> <td style="text-align: center;">\$280</td> <td style="text-align: right;">\$ <u>570.00</u></td> </tr> <tr> <td><input type="checkbox"/> Three months (37 CFR 1.17(a)(3))</td> <td style="text-align: center;">\$1270</td> <td style="text-align: center;">\$635</td> <td style="text-align: right;">\$ _____</td> </tr> <tr> <td><input type="checkbox"/> Four months (37 CFR 1.17(a)(4))</td> <td style="text-align: center;">\$1980</td> <td style="text-align: center;">\$990</td> <td style="text-align: right;">\$ _____</td> </tr> <tr> <td><input type="checkbox"/> Five months (37 CFR 1.17(a)(5))</td> <td style="text-align: center;">\$2690</td> <td style="text-align: center;">\$1345</td> <td style="text-align: right;">\$ _____</td> </tr> </tbody> </table> <p><input type="checkbox"/> Applicant claims small entity status. See 37 CFR 1.27.</p> <p><input type="checkbox"/> A check in the amount of the fee is enclosed.</p> <p><input type="checkbox"/> Payment by credit card. Form PTO-2038 is attached.</p> <p><input type="checkbox"/> The Director has already been authorized to charge fees in this application to a Deposit Account.</p> <p><input checked="" type="checkbox"/> The Director is hereby authorized to charge any fees which may be required, or credit any overpayment, to Deposit Account Number <u>190733</u>.</p> <p><b>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.</b></p> <p>I am the <input type="checkbox"/> applicant/inventor.</p> <p><input type="checkbox"/> assignee of record of the entire interest. See 37 CFR 3.71.<br/>Statement under 37 CFR 3.73(b) is enclosed (Form PTO/SB/96).</p> <p><input checked="" type="checkbox"/> attorney or agent of record. Registration Number <u>32,141</u></p> <p><input type="checkbox"/> attorney or agent under 37 CFR 1.34.<br/>Registration number if acting under 37 CFR 1.34 _____</p> <p style="margin-left: 40px;">/Sarah A. Kagan/ <span style="float: right;">11 March 2013</span></p> <hr style="width: 50%; margin-left: 0;"/> <p style="margin-left: 100px; text-align: center;">Signature <span style="float: right;">Date</span></p> <p style="margin-left: 40px;">Sarah A. Kagan <span style="float: right;">(202) 824-3000</span></p> <hr style="width: 50%; margin-left: 0;"/> <p style="margin-left: 100px; text-align: center;">Typed or printed name <span style="float: right;">Telephone Number</span></p> <p>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.</p> <p><input checked="" type="checkbox"/> Total of <u>1</u> forms are submitted.</p> |                                          |                         | <u>Fee</u>       | <u>Small Entity Fee</u> |  | <input type="checkbox"/> One month (37 CFR 1.17(a)(1)) | \$150 | \$75 | \$ _____ | <input checked="" type="checkbox"/> Two months (37 CFR 1.17(a)(2)) | \$560 | \$280 | \$ <u>570.00</u> | <input type="checkbox"/> Three months (37 CFR 1.17(a)(3)) | \$1270 | \$635 | \$ _____ | <input type="checkbox"/> Four months (37 CFR 1.17(a)(4)) | \$1980 | \$990 | \$ _____ | <input type="checkbox"/> Five months (37 CFR 1.17(a)(5)) | \$2690 | \$1345 | \$ _____ |
|                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                    | <u>Fee</u>                               | <u>Small Entity Fee</u> |                  |                         |  |                                                        |       |      |          |                                                                    |       |       |                  |                                                           |        |       |          |                                                          |        |       |          |                                                          |        |        |          |
| <input type="checkbox"/> One month (37 CFR 1.17(a)(1))                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                             | \$150                                    | \$75                    | \$ _____         |                         |  |                                                        |       |      |          |                                                                    |       |       |                  |                                                           |        |       |          |                                                          |        |       |          |                                                          |        |        |          |
| <input checked="" type="checkbox"/> Two months (37 CFR 1.17(a)(2))                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                 | \$560                                    | \$280                   | \$ <u>570.00</u> |                         |  |                                                        |       |      |          |                                                                    |       |       |                  |                                                           |        |       |          |                                                          |        |       |          |                                                          |        |        |          |
| <input type="checkbox"/> Three months (37 CFR 1.17(a)(3))                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                          | \$1270                                   | \$635                   | \$ _____         |                         |  |                                                        |       |      |          |                                                                    |       |       |                  |                                                           |        |       |          |                                                          |        |       |          |                                                          |        |        |          |
| <input type="checkbox"/> Four months (37 CFR 1.17(a)(4))                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                           | \$1980                                   | \$990                   | \$ _____         |                         |  |                                                        |       |      |          |                                                                    |       |       |                  |                                                           |        |       |          |                                                          |        |       |          |                                                          |        |        |          |
| <input type="checkbox"/> Five months (37 CFR 1.17(a)(5))                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                           | \$2690                                   | \$1345                  | \$ _____         |                         |  |                                                        |       |      |          |                                                                    |       |       |                  |                                                           |        |       |          |                                                          |        |       |          |                                                          |        |        |          |

This collection of information is required by 37 CFR 1.136(a). 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 6 minutes to complete, including gathering, preparing, and submitting the completed application form to the USPTO. Time will vary depending upon the individual case. Any comments on the amount of time you require to complete this form and/or suggestions for reducing this burden, should be sent to the Chief Information Officer, U.S. Patent and Trademark Office, U.S. Department of Commerce, P.O. Box 1450, Alexandria, VA 22313-1450. DO NOT SEND FEES OR COMPLETED FORMS TO THIS ADDRESS. **SEND TO: Commissioner for Patents, P.O. Box 1450, Alexandria, VA 22313-1450.**

*If you need assistance in completing the form, call 1-800-PTO-9199 and select option 2.*

## 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

|                                                |                                    |                 |               |                             |
|------------------------------------------------|------------------------------------|-----------------|---------------|-----------------------------|
| <b>Application Number:</b>                     | 13071105                           |                 |               |                             |
| <b>Filing Date:</b>                            | 24-Mar-2011                        |                 |               |                             |
| <b>Title of Invention:</b>                     | Digital Amplification              |                 |               |                             |
| <b>First Named Inventor/Applicant Name:</b>    | Bert VOGELSTEIN                    |                 |               |                             |
| <b>Filer:</b>                                  | Sarah Anne Kagan./Jennifer Hazzard |                 |               |                             |
| <b>Attorney Docket Number:</b>                 | 001107.00866                       |                 |               |                             |
| Filed as Large Entity                          |                                    |                 |               |                             |
| <b>Utility under 35 USC 111(a) Filing Fees</b> |                                    |                 |               |                             |
| <b>Description</b>                             | <b>Fee Code</b>                    | <b>Quantity</b> | <b>Amount</b> | <b>Sub-Total in USD(\$)</b> |
| <b>Basic Filing:</b>                           |                                    |                 |               |                             |
| <b>Pages:</b>                                  |                                    |                 |               |                             |
| <b>Claims:</b>                                 |                                    |                 |               |                             |
| <b>Miscellaneous-Filing:</b>                   |                                    |                 |               |                             |
| <b>Petition:</b>                               |                                    |                 |               |                             |
| <b>Patent-Appeals-and-Interference:</b>        |                                    |                 |               |                             |
| <b>Post-Allowance-and-Post-Issuance:</b>       |                                    |                 |               |                             |
| <b>Extension-of-Time:</b>                      |                                    |                 |               |                             |
| Extension - 2 months with \$0 paid             | 1252                               | 1               | 570           | 570                         |

| Description              | Fee Code | Quantity | Amount | Sub-Total in USD(\$) |
|--------------------------|----------|----------|--------|----------------------|
| <b>Miscellaneous:</b>    |          |          |        |                      |
| <b>Total in USD (\$)</b> |          |          |        | <b>570</b>           |

## Electronic Acknowledgement Receipt

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

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**File Listing:**

| Document Number                                            | Document Description                                  | File Name                     | File Size(Bytes)/<br>Message Digest                | Multi Part /.zip | Pages (if appl.) |
|------------------------------------------------------------|-------------------------------------------------------|-------------------------------|----------------------------------------------------|------------------|------------------|
| 1                                                          |                                                       | Response-to-NFOA-as-filed.PDF | 95962<br>c2b10b9891e55742fa615f10b56cd60a6bc4bf0a  | yes              | 8                |
| <b>Multipart Description/PDF files in .zip description</b> |                                                       |                               |                                                    |                  |                  |
|                                                            | <b>Document Description</b>                           |                               | <b>Start</b>                                       |                  | <b>End</b>       |
|                                                            | Amendment/Req. Reconsideration-After Non-Final Reject |                               | 1                                                  |                  | 1                |
|                                                            | Claims                                                |                               | 2                                                  |                  | 5                |
|                                                            | Applicant Arguments/Remarks Made in an Amendment      |                               | 6                                                  |                  | 8                |
| <b>Warnings:</b>                                           |                                                       |                               |                                                    |                  |                  |
| <b>Information:</b>                                        |                                                       |                               |                                                    |                  |                  |
| 2                                                          | Extension of Time                                     | Petition-for-EOT.PDF          | 289378<br>8919f47c7ad4711247bcaa1cf63e1efecb836292 | no               | 2                |
| <b>Warnings:</b>                                           |                                                       |                               |                                                    |                  |                  |
| <b>Information:</b>                                        |                                                       |                               |                                                    |                  |                  |
| 3                                                          | Fee Worksheet (SB06)                                  | fee-info.pdf                  | 30272<br>92ce82a7a1eb8b0196183f2eccec64f34b3c693e2 | no               | 2                |
| <b>Warnings:</b>                                           |                                                       |                               |                                                    |                  |                  |
| <b>Information:</b>                                        |                                                       |                               |                                                    |                  |                  |
| <b>Total Files Size (in bytes):</b>                        |                                                       |                               | 415612                                             |                  |                  |

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

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

**National Stage of an International Application under 35 U.S.C. 371**

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

**New International Application Filed with the USPTO as a Receiving Office**

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



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|                                                                                              |                                                                                                                                                                                                                               |                                  |                                    |               |                                                   |                                  |                                       |                 |                     |  |
|----------------------------------------------------------------------------------------------|-------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------|----------------------------------|------------------------------------|---------------|---------------------------------------------------|----------------------------------|---------------------------------------|-----------------|---------------------|--|
| <b>PATENT APPLICATION FEE DETERMINATION RECORD</b><br>Substitute for Form PTO-875            |                                                                                                                                                                                                                               |                                  |                                    |               | Application or Docket Number<br><b>13/071,105</b> | Filing Date<br><b>03/24/2011</b> | <input type="checkbox"/> To be Mailed |                 |                     |  |
| <b>APPLICATION AS FILED – PART I</b>                                                         |                                                                                                                                                                                                                               |                                  |                                    |               | SMALL ENTITY <input type="checkbox"/> OR          |                                  | OTHER THAN SMALL ENTITY               |                 |                     |  |
| (Column 1)                                                                                   |                                                                                                                                                                                                                               | (Column 2)                       |                                    |               |                                                   |                                  |                                       |                 |                     |  |
| FOR                                                                                          | NUMBER FILED                                                                                                                                                                                                                  | NUMBER EXTRA                     |                                    |               | RATE (\$)                                         | FEE (\$)                         |                                       | RATE (\$)       | FEE (\$)            |  |
| <input type="checkbox"/> BASIC FEE<br><small>(37 CFR 1.16(a), (b), or (c))</small>           | N/A                                                                                                                                                                                                                           | N/A                              |                                    |               | N/A                                               |                                  |                                       | N/A             |                     |  |
| <input type="checkbox"/> SEARCH FEE<br><small>(37 CFR 1.16(k), (l), or (m))</small>          | N/A                                                                                                                                                                                                                           | N/A                              |                                    |               | N/A                                               |                                  |                                       | N/A             |                     |  |
| <input type="checkbox"/> EXAMINATION FEE<br><small>(37 CFR 1.16(o), (p), or (q))</small>     | N/A                                                                                                                                                                                                                           | N/A                              |                                    |               | N/A                                               |                                  |                                       | N/A             |                     |  |
| TOTAL CLAIMS<br><small>(37 CFR 1.16(i))</small>                                              | minus 20 =                                                                                                                                                                                                                    | *                                |                                    |               | X \$ =                                            |                                  | OR                                    | X \$ =          |                     |  |
| INDEPENDENT CLAIMS<br><small>(37 CFR 1.16(h))</small>                                        | minus 3 =                                                                                                                                                                                                                     | *                                |                                    |               | X \$ =                                            |                                  |                                       | X \$ =          |                     |  |
| <input type="checkbox"/> APPLICATION SIZE FEE<br><small>(37 CFR 1.16(s))</small>             | 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). |                                  |                                    |               |                                                   |                                  |                                       |                 |                     |  |
| <input type="checkbox"/> MULTIPLE DEPENDENT CLAIM PRESENT<br><small>(37 CFR 1.16(j))</small> |                                                                                                                                                                                                                               |                                  |                                    |               |                                                   |                                  |                                       |                 |                     |  |
| * If the difference in column 1 is less than zero, enter "0" in column 2.                    |                                                                                                                                                                                                                               |                                  |                                    |               | TOTAL                                             |                                  |                                       | TOTAL           |                     |  |
| <b>APPLICATION AS AMENDED – PART II</b>                                                      |                                                                                                                                                                                                                               |                                  |                                    |               | SMALL ENTITY OR                                   |                                  | OTHER THAN SMALL ENTITY               |                 |                     |  |
| (Column 1)                                                                                   |                                                                                                                                                                                                                               | (Column 2)                       |                                    | (Column 3)    |                                                   |                                  |                                       |                 |                     |  |
| AMENDMENT                                                                                    | <b>03/11/2013</b>                                                                                                                                                                                                             | CLAIMS REMAINING AFTER AMENDMENT | HIGHEST NUMBER PREVIOUSLY PAID FOR | PRESENT EXTRA | RATE (\$)                                         | ADDITIONAL FEE (\$)              | OR                                    | RATE (\$)       | ADDITIONAL FEE (\$) |  |
|                                                                                              | Total (37 CFR 1.16(i))                                                                                                                                                                                                        | * 20                             | Minus                              | ** 68         | =                                                 | 0                                | OR                                    | X \$62=         | 0                   |  |
|                                                                                              | Independent (37 CFR 1.16(h))                                                                                                                                                                                                  | * 1                              | Minus                              | ***6          | =                                                 | 0                                | OR                                    | X \$250=        | 0                   |  |
|                                                                                              | <input type="checkbox"/> Application Size Fee (37 CFR 1.16(s))                                                                                                                                                                |                                  |                                    |               |                                                   |                                  |                                       |                 |                     |  |
|                                                                                              | <input type="checkbox"/> FIRST PRESENTATION OF MULTIPLE DEPENDENT CLAIM (37 CFR 1.16(j))                                                                                                                                      |                                  |                                    |               |                                                   |                                  | OR                                    |                 |                     |  |
|                                                                                              |                                                                                                                                                                                                                               |                                  |                                    |               | TOTAL ADD'L FEE                                   |                                  | OR                                    | TOTAL ADD'L FEE | <b>0</b>            |  |
| AMENDMENT                                                                                    | Total (37 CFR 1.16(i))                                                                                                                                                                                                        | *                                | Minus                              | **            | =                                                 |                                  | OR                                    | X \$ =          |                     |  |
|                                                                                              | Independent (37 CFR 1.16(h))                                                                                                                                                                                                  | *                                | Minus                              | ***           | =                                                 |                                  | OR                                    | X \$ =          |                     |  |
|                                                                                              | <input type="checkbox"/> Application Size Fee (37 CFR 1.16(s))                                                                                                                                                                |                                  |                                    |               |                                                   |                                  |                                       |                 |                     |  |
|                                                                                              | <input type="checkbox"/> 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/

This collection of information is required by 37 CFR 1.16. The information is required to obtain or retain a benefit by the public which is to file (and by the USPTO to process) an application. Confidentiality is governed by 35 U.S.C. 122 and 37 CFR 1.14. This collection is estimated to take 12 minutes to complete, including gathering, preparing, and submitting the completed application form to the USPTO. Time will vary depending upon the individual case. Any comments on the amount of time you require to complete this form and/or suggestions for reducing this burden, should be sent to the Chief Information Officer, U.S. Patent and Trademark Office, U.S. Department of Commerce, P.O. Box 1450, Alexandria, VA 22313-1450. DO NOT SEND FEES OR COMPLETED FORMS TO THIS ADDRESS. **SEND TO: Commissioner for Patents, P.O. Box 1450, Alexandria, VA 22313-1450.**

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Table with columns: 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
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
10/10/2012 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.

|                              |                                      |                                          |  |
|------------------------------|--------------------------------------|------------------------------------------|--|
| <b>Office Action Summary</b> | <b>Application No.</b><br>13/071,105 | <b>Applicant(s)</b><br>VOGELSTEIN ET AL. |  |
|                              | <b>Examiner</b><br>SAMUEL WOOLWINE   | <b>Art Unit</b><br>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) <input checked="" type="checkbox"/> Notice of References Cited (PTO-892)                                                         | 4) <input type="checkbox"/> Interview Summary (PTO-413)<br>Paper No(s)/Mail Date. _____ |
| 2) <input type="checkbox"/> Notice of Draftsperson's Patent Drawing Review (PTO-948)                                                | 5) <input type="checkbox"/> Notice of Informal Patent Application                       |
| 3) <input checked="" type="checkbox"/> Information Disclosure Statement(s) (PTO/SB/08)<br>Paper No(s)/Mail Date <u>03/24/2011</u> . | 6) <input type="checkbox"/> 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:

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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.

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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 negated 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

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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

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| 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 | <input type="checkbox"/> |
| 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                                                                                                                | <input type="checkbox"/> |
| 19 | R. PARSONS ET AL., "Mismatch Repair Deficiency in Phenotypically Normal Human Cells," Science, May 5, 1995, Vol. 268, pp. 738-740                                                                                                                                                      | <input type="checkbox"/> |

|                                                                                                     |                        |                        |              |
|-----------------------------------------------------------------------------------------------------|------------------------|------------------------|--------------|
| <b>INFORMATION DISCLOSURE<br/>STATEMENT BY APPLICANT</b><br>( 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                                         | <input type="checkbox"/> |
| 21 | WHITCOMB ET AL., "Detection of PCR Products Using Self-Probing Amplicons and Fluorescence," Nature Biotechnology, August 1999, Vol. 17, pp. 804-807                                                              | <input type="checkbox"/> |
| 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        | <input type="checkbox"/> |
| 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 | <input type="checkbox"/> |
| 24 | Notice of Reasons for Rejection dispatched April 28, 2010 in Japanese Application No. 2001-513641 and English translation thereof.                                                                               | <input type="checkbox"/> |
| 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).                        | <input type="checkbox"/> |
| 26 | NEWTON, PCR Essential Data, pages 51-52, 1995                                                                                                                                                                    | <input type="checkbox"/> |
| 27 | Office Action dated June 11, 2010, in co-pending application 11/709,742                                                                                                                                          | <input type="checkbox"/> |
| 28 | Office Action dated December 29, 2009 in co-pending application 11/709,742                                                                                                                                       | <input type="checkbox"/> |
| 29 | Office Action dated September 18, 2009 in co-pending application 11/709,742                                                                                                                                      | <input type="checkbox"/> |
| 30 | Office Action dated June 5, 2009 in co-pending application 11/709,742                                                                                                                                            | <input type="checkbox"/> |

**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 SIGNATURE**

|                    |                   |                 |            |
|--------------------|-------------------|-----------------|------------|
| Examiner Signature | /Samuel Woolwine/ | Date Considered | 10/02/2012 |
|--------------------|-------------------|-----------------|------------|

\*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.

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

## 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;<br>USPAT;<br>USOCR;<br>FPRS; EPO;<br>JPO;<br>DERWENT;<br>IBM_TDB | OR               | ON      | 2012/10/02<br>15:01 |
| L2    | 361   | l1 and (@ad<"19990802"<br>@pd<"19990802")                                                                                                                                                                      | US-PGPUB;<br>USPAT;<br>USOCR;<br>FPRS; EPO;<br>JPO;<br>DERWENT;<br>IBM_TDB | OR               | ON      | 2012/10/02<br>15:01 |
| L3    | 12232 | rare near5 (sequence target mutation variant variation polymorphism)                                                                                                                                           | US-PGPUB;<br>USPAT;<br>USOCR;<br>FPRS; EPO;<br>JPO;<br>DERWENT;<br>IBM_TDB | OR               | ON      | 2012/10/02<br>15:02 |
| L4    | 14    | l2 and l3                                                                                                                                                                                                      | US-PGPUB;<br>USPAT;<br>USOCR;<br>FPRS; EPO;<br>JPO;<br>DERWENT;<br>IBM_TDB | OR               | ON      | 2012/10/02<br>15:02 |
| S2    | 4     | ("7915015" "7824889" "6753147"<br>"6440706").PN.                                                                                                                                                               | USPAT                                                                      | OR               | OFF     | 2012/10/01<br>07:49 |
| S3    | 1     | ("20080287318").PN.                                                                                                                                                                                            | US-PGPUB;<br>USPAT                                                         | OR               | OFF     | 2012/10/01<br>15:54 |
| S4    | 1132  | "limiting dilution" same pcr                                                                                                                                                                                   | US-PGPUB;<br>USPAT;<br>USOCR;<br>FPRS; EPO;<br>JPO;<br>DERWENT;<br>IBM_TDB | OR               | OFF     | 2012/10/01<br>16:52 |
| S5    | 123   | S4 and (@ad<"19990802"<br>@pd<"19990802")                                                                                                                                                                      | US-PGPUB;<br>USPAT;<br>USOCR;<br>FPRS; EPO;<br>JPO;<br>DERWENT;<br>IBM_TDB | OR               | OFF     | 2012/10/01<br>17:04 |
| S6    | 85    | S5 and sequencing                                                                                                                                                                                              | US-PGPUB;<br>USPAT;<br>USOCR;<br>FPRS; EPO;                                | OR               | OFF     | 2012/10/01<br>17:04 |



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

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

**10/ 2/ 2012 3:45:50 PM**

**C:\Users\swoolwine\Documents\EAST\Workspaces\13071105.wsp**

**EAST Search History****EAST Search History (Prior Art)**

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

10/ 2/ 2012 5:51:13 PM

C:\Users\swoolwine\Documents\EAST\Workspaces\13071105.wsp

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: <b>Digital Amplification</b> | ) | 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 is 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 ~~±~~ 49 wherein each of the assay samples has on average 0.5 molecules of template.

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

52. (Currently amended) 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 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 ~~±~~ 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 ~~±~~ 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 ~~±~~ 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 ~~±~~ 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 ~~±~~ 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 ~~4~~ 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 ~~4~~ 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 ~~4~~ 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 ~~4~~ 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 ~~4~~ 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 ~~4~~ 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 ~~4~~ 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 ~~4~~ 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 ~~4~~ 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

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|                                                                                                                                                                                                       |                                  |                                          |                         |
|-------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------|----------------------------------|------------------------------------------|-------------------------|
| <b>PETITION FOR EXTENSION OF TIME UNDER 37 CFR 1.136(a)</b>                                                                                                                                           |                                  | Docket Number (Optional)<br>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):                                                                                       |                                  |                                          |                         |
|                                                                                                                                                                                                       |                                  | <u>Fee</u>                               | <u>Small Entity Fee</u> |
| <input checked="" type="checkbox"/>                                                                                                                                                                   | One month (37 CFR 1.17(a)(1))    | \$150                                    | \$75 \$ <u>150.00</u>   |
| <input type="checkbox"/>                                                                                                                                                                              | Two months (37 CFR 1.17(a)(2))   | \$560                                    | \$280 \$ _____          |
| <input type="checkbox"/>                                                                                                                                                                              | Three months (37 CFR 1.17(a)(3)) | \$1270                                   | \$635 \$ _____          |
| <input type="checkbox"/>                                                                                                                                                                              | Four months (37 CFR 1.17(a)(4))  | \$1980                                   | \$990 \$ _____          |
| <input type="checkbox"/>                                                                                                                                                                              | Five months (37 CFR 1.17(a)(5))  | \$2690                                   | \$1345 \$ _____         |
| <input type="checkbox"/> Applicant claims small entity status. See 37 CFR 1.27.                                                                                                                       |                                  |                                          |                         |
| <input type="checkbox"/> A check in the amount of the fee is enclosed.                                                                                                                                |                                  |                                          |                         |
| <input type="checkbox"/> Payment by credit card. Form PTO-2038 is attached.                                                                                                                           |                                  |                                          |                         |
| <input type="checkbox"/> The Director has already been authorized to charge fees in this application to a Deposit Account.                                                                            |                                  |                                          |                         |
| <input checked="" type="checkbox"/> The Director is hereby authorized to charge any fees which may be required, or credit any overpayment, to Deposit Account Number <u>190733</u> .                  |                                  |                                          |                         |
| <b>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.</b>               |                                  |                                          |                         |
| I am the <input type="checkbox"/> applicant/inventor.                                                                                                                                                 |                                  |                                          |                         |
| <input type="checkbox"/> assignee of record of the entire interest. See 37 CFR 3.71.<br>Statement under 37 CFR 3.73(b) is enclosed (Form PTO/SB/96).                                                  |                                  |                                          |                         |
| <input checked="" type="checkbox"/> attorney or agent of record. Registration Number <u>32141</u>                                                                                                     |                                  |                                          |                         |
| <input type="checkbox"/> attorney or agent under 37 CFR 1.34.<br>Registration number if acting under 37 CFR 1.34 _____                                                                                |                                  |                                          |                         |
| <u>/Sarah A. Kagan/</u>                                                                                                                                                                               |                                  | <u>June 11, 2012</u>                     |                         |
| Signature                                                                                                                                                                                             |                                  | Date                                     |                         |
| <u>Sarah A. Kagan</u>                                                                                                                                                                                 |                                  | <u>(202) 824-3000</u>                    |                         |
| Typed or printed name                                                                                                                                                                                 |                                  | 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. |                                  |                                          |                         |
| <input checked="" type="checkbox"/> Total of <u>1</u> forms are submitted.                                                                                                                            |                                  |                                          |                         |

This collection of information is required by 37 CFR 1.136(a). 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 6 minutes to complete, including gathering, preparing, and submitting the completed application form to the USPTO. Time will vary depending upon the individual case. Any comments on the amount of time you require to complete this form and/or suggestions for reducing this burden, should be sent to the Chief Information Officer, U.S. Patent and Trademark Office, U.S. Department of Commerce, P.O. Box 1450, Alexandria, VA 22313-1450. DO NOT SEND FEES OR COMPLETED FORMS TO THIS ADDRESS. **SEND TO: Commissioner for Patents, P.O. Box 1450, Alexandria, VA 22313-1450.**

If you need assistance in completing the form, call 1-800-PTO-9199 and select option 2.

## 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

|                                                |                                 |                 |               |                             |
|------------------------------------------------|---------------------------------|-----------------|---------------|-----------------------------|
| <b>Application Number:</b>                     | 13071105                        |                 |               |                             |
| <b>Filing Date:</b>                            | 24-Mar-2011                     |                 |               |                             |
| <b>Title of Invention:</b>                     | Digital Amplification           |                 |               |                             |
| <b>First Named Inventor/Applicant Name:</b>    | Bert VOGELSTEIN                 |                 |               |                             |
| <b>Filer:</b>                                  | Sarah Anne Kagan./Leatrice sims |                 |               |                             |
| <b>Attorney Docket Number:</b>                 | 001107.00866                    |                 |               |                             |
| Filed as Large Entity                          |                                 |                 |               |                             |
| <b>Utility under 35 USC 111(a) Filing Fees</b> |                                 |                 |               |                             |
| <b>Description</b>                             | <b>Fee Code</b>                 | <b>Quantity</b> | <b>Amount</b> | <b>Sub-Total in USD(\$)</b> |
| <b>Basic Filing:</b>                           |                                 |                 |               |                             |
| <b>Pages:</b>                                  |                                 |                 |               |                             |
| <b>Claims:</b>                                 |                                 |                 |               |                             |
| <b>Miscellaneous-Filing:</b>                   |                                 |                 |               |                             |
| <b>Petition:</b>                               |                                 |                 |               |                             |
| <b>Patent-Appeals-and-Interference:</b>        |                                 |                 |               |                             |
| <b>Post-Allowance-and-Post-Issuance:</b>       |                                 |                 |               |                             |
| <b>Extension-of-Time:</b>                      |                                 |                 |               |                             |
| Extension - 1 month with \$0 paid              | 1251                            | 1               | 150           | 150                         |

| Description              | Fee Code | Quantity | Amount | Sub-Total in USD(\$) |
|--------------------------|----------|----------|--------|----------------------|
| <b>Miscellaneous:</b>    |          |          |        |                      |
| <b>Total in USD (\$)</b> |          |          |        | <b>150</b>           |

## Electronic Acknowledgement Receipt

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

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| 1                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                           |                      | response_to_RR.pdf                       | 83294<br>abfb45b23bf7dbec6e6d49bd5fb4b0d6ae1758080 | yes        | 10 |
| <b>Multipart Description/PDF files in .zip description</b>                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                  |                      |                                          |                                                    |            |    |
|                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                             |                      | <b>Document Description</b>              | <b>Start</b>                                       | <b>End</b> |    |
|                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                             |                      | Response to Election / Restriction Filed | 1                                                  | 1          |    |
|                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                             |                      | Claims                                   | 2                                                  | 10         |    |
| <b>Warnings:</b>                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                            |                      |                                          |                                                    |            |    |
| <b>Information:</b>                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                         |                      |                                          |                                                    |            |    |
| 2                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                           | Extension of Time    | EOT_filed_with_RR.pdf                    | 286857<br>1f2225aaa12244d9c218066b5f8025f32bfb15fb | no         | 2  |
| <b>Warnings:</b>                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                            |                      |                                          |                                                    |            |    |
| <b>Information:</b>                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                         |                      |                                          |                                                    |            |    |
| 3                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                           | Fee Worksheet (SB06) | fee-info.pdf                             | 30091<br>07c17696daf0ce93600409d2e67488efe9da944   | no         | 2  |
| <b>Warnings:</b>                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                            |                      |                                          |                                                    |            |    |
| <b>Information:</b>                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                         |                      |                                          |                                                    |            |    |
| <b>Total Files Size (in bytes):</b>                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                         |                      |                                          | 400242                                             |            |    |
| <p><b>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.</b></p> <p><b><u>New Applications Under 35 U.S.C. 111</u></b><br/> <b>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.</b></p> <p><b><u>National Stage of an International Application under 35 U.S.C. 371</u></b><br/> <b>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.</b></p> <p><b><u>New International Application Filed with the USPTO as a Receiving Office</u></b><br/> <b>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.</b></p> |                      |                                          |                                                    |            |    |

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|                                                                                                                                 |                                                                                                                                                                                                                               |                                  |       |                                    |                                                                  |                 |                                  |            |                                       |                     |
|---------------------------------------------------------------------------------------------------------------------------------|-------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------|----------------------------------|-------|------------------------------------|------------------------------------------------------------------|-----------------|----------------------------------|------------|---------------------------------------|---------------------|
| <b>PATENT APPLICATION FEE DETERMINATION RECORD</b><br>Substitute for Form PTO-875                                               |                                                                                                                                                                                                                               |                                  |       |                                    | Application or Docket Number<br><b>13/071,105</b>                |                 | Filing Date<br><b>03/24/2011</b> |            | <input type="checkbox"/> To be Mailed |                     |
| <b>APPLICATION AS FILED – PART I</b>                                                                                            |                                                                                                                                                                                                                               |                                  |       |                                    | SMALL ENTITY <input type="checkbox"/> OR OTHER THAN SMALL ENTITY |                 |                                  |            |                                       |                     |
| (Column 1)                                                                                                                      |                                                                                                                                                                                                                               | (Column 2)                       |       |                                    | (Column 3)                                                       |                 | (Column 4)                       |            |                                       |                     |
| FOR                                                                                                                             | NUMBER FILED                                                                                                                                                                                                                  | NUMBER EXTRA                     |       |                                    | RATE (\$)                                                        | FEE (\$)        | OR                               | RATE (\$)  | FEE (\$)                              |                     |
| <input type="checkbox"/> BASIC FEE<br><small>(37 CFR 1.16(a), (b), or (c))</small>                                              | N/A                                                                                                                                                                                                                           | N/A                              |       |                                    | N/A                                                              |                 | OR                               | N/A        |                                       |                     |
| <input type="checkbox"/> SEARCH FEE<br><small>(37 CFR 1.16(k), (l), or (m))</small>                                             | N/A                                                                                                                                                                                                                           | N/A                              |       |                                    | N/A                                                              |                 | OR                               | N/A        |                                       |                     |
| <input type="checkbox"/> EXAMINATION FEE<br><small>(37 CFR 1.16(o), (p), or (q))</small>                                        | N/A                                                                                                                                                                                                                           | N/A                              |       |                                    | N/A                                                              |                 | OR                               | N/A        |                                       |                     |
| TOTAL CLAIMS<br><small>(37 CFR 1.16(i))</small>                                                                                 | minus 20 =                                                                                                                                                                                                                    | *                                |       |                                    | X \$ =                                                           |                 | OR                               | X \$ =     |                                       |                     |
| INDEPENDENT CLAIMS<br><small>(37 CFR 1.16(h))</small>                                                                           | minus 3 =                                                                                                                                                                                                                     | *                                |       |                                    | X \$ =                                                           |                 | OR                               | X \$ =     |                                       |                     |
| <input type="checkbox"/> APPLICATION SIZE FEE<br><small>(37 CFR 1.16(s))</small>                                                | 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). |                                  |       |                                    |                                                                  |                 |                                  |            |                                       |                     |
| <input type="checkbox"/> MULTIPLE DEPENDENT CLAIM PRESENT <small>(37 CFR 1.16(j))</small>                                       |                                                                                                                                                                                                                               |                                  |       |                                    |                                                                  |                 |                                  |            |                                       |                     |
| * If the difference in column 1 is less than zero, enter "0" in column 2.                                                       |                                                                                                                                                                                                                               |                                  |       |                                    | TOTAL                                                            |                 | OR                               | TOTAL      |                                       |                     |
| <b>APPLICATION AS AMENDED – PART II</b>                                                                                         |                                                                                                                                                                                                                               |                                  |       |                                    | SMALL ENTITY OR OTHER THAN SMALL ENTITY                          |                 |                                  |            |                                       |                     |
| (Column 1)                                                                                                                      |                                                                                                                                                                                                                               | (Column 2)                       |       | (Column 3)                         |                                                                  | (Column 4)      |                                  | (Column 5) |                                       |                     |
| AMENDMENT                                                                                                                       | DATE                                                                                                                                                                                                                          | CLAIMS REMAINING AFTER AMENDMENT | MINUS | HIGHEST NUMBER PREVIOUSLY PAID FOR | PRESENT EXTRA                                                    | RATE (\$)       | ADDITIONAL FEE (\$)              | OR         | RATE (\$)                             | ADDITIONAL FEE (\$) |
|                                                                                                                                 | <b>06/11/2012</b>                                                                                                                                                                                                             | *                                | Minus | ** 68                              | = 0                                                              | X \$ =          |                                  | OR         | X \$60=                               | 0                   |
|                                                                                                                                 | Total <small>(37 CFR 1.16(i))</small>                                                                                                                                                                                         | * 68                             | Minus | ** 68                              | = 0                                                              | X \$ =          |                                  | OR         | X \$250=                              | 0                   |
|                                                                                                                                 | Independent <small>(37 CFR 1.16(b))</small>                                                                                                                                                                                   | * 6                              | Minus | ***6                               | = 0                                                              |                 |                                  | OR         |                                       |                     |
| <input type="checkbox"/> Application Size Fee <small>(37 CFR 1.16(s))</small>                                                   |                                                                                                                                                                                                                               |                                  |       |                                    |                                                                  |                 |                                  |            |                                       |                     |
| <input type="checkbox"/> FIRST PRESENTATION OF MULTIPLE DEPENDENT CLAIM <small>(37 CFR 1.16(j))</small>                         |                                                                                                                                                                                                                               |                                  |       |                                    |                                                                  |                 |                                  |            |                                       |                     |
|                                                                                                                                 |                                                                                                                                                                                                                               |                                  |       |                                    |                                                                  | TOTAL ADD'L FEE |                                  | OR         | TOTAL ADD'L FEE                       | <b>0</b>            |
| (Column 1)                                                                                                                      |                                                                                                                                                                                                                               | (Column 2)                       |       | (Column 3)                         |                                                                  | (Column 4)      |                                  | (Column 5) |                                       |                     |
| AMENDMENT                                                                                                                       | DATE                                                                                                                                                                                                                          | CLAIMS REMAINING AFTER AMENDMENT | MINUS | HIGHEST NUMBER PREVIOUSLY PAID FOR | PRESENT EXTRA                                                    | RATE (\$)       | ADDITIONAL FEE (\$)              | OR         | RATE (\$)                             | ADDITIONAL FEE (\$) |
|                                                                                                                                 |                                                                                                                                                                                                                               | *                                | Minus | **                                 | =                                                                | X \$ =          |                                  | OR         | X \$ =                                |                     |
|                                                                                                                                 | Total <small>(37 CFR 1.16(i))</small>                                                                                                                                                                                         | *                                | Minus | **                                 | =                                                                | X \$ =          |                                  | OR         | X \$ =                                |                     |
|                                                                                                                                 | Independent <small>(37 CFR 1.16(b))</small>                                                                                                                                                                                   | *                                | Minus | ***                                | =                                                                |                 |                                  | OR         |                                       |                     |
| <input type="checkbox"/> Application Size Fee <small>(37 CFR 1.16(s))</small>                                                   |                                                                                                                                                                                                                               |                                  |       |                                    |                                                                  |                 |                                  |            |                                       |                     |
| <input type="checkbox"/> FIRST PRESENTATION OF MULTIPLE DEPENDENT CLAIM <small>(37 CFR 1.16(j))</small>                         |                                                                                                                                                                                                                               |                                  |       |                                    |                                                                  |                 |                                  |            |                                       |                     |
|                                                                                                                                 |                                                                                                                                                                                                                               |                                  |       |                                    |                                                                  | 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.                                           |                                                                                                                                                                                                                               |                                  |       |                                    | <b>Legal Instrument Examiner:</b><br><b>/KIM P. DOZIER/</b>      |                 |                                  |            |                                       |                     |
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This collection of information is required by 37 CFR 1.16. The information is required to obtain or retain a benefit by the public which is to file (and by the USPTO to process) an application. Confidentiality is governed by 35 U.S.C. 122 and 37 CFR 1.14. This collection is estimated to take 12 minutes to complete, including gathering, preparing, and submitting the completed application form to the USPTO. Time will vary depending upon the individual case. Any comments on the amount of time you require to complete this form and/or suggestions for reducing this burden, should be sent to the Chief Information Officer, U.S. Patent and Trademark Office, U.S. Department of Commerce, P.O. Box 1450, Alexandria, VA 22313-1450. DO NOT SEND FEES OR COMPLETED FORMS TO THIS ADDRESS. **SEND TO: Commissioner for Patents, P.O. Box 1450, Alexandria, VA 22313-1450.**

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| APPLICATION NO.                                                                                                           | FILING DATE | FIRST NAMED INVENTOR | ATTORNEY DOCKET NO. | CONFIRMATION NO. |
|---------------------------------------------------------------------------------------------------------------------------|-------------|----------------------|---------------------|------------------|
| 13/071,105                                                                                                                | 03/24/2011  | Bert VOGELSTEIN      | 001107.00866        | 3361             |
| 11332                                                                                                                     | 7590        | 04/10/2012           | EXAMINER            |                  |
| Banner & Witcoff, Ltd.<br>Attorneys for client 001107<br>1100 13th Street N.W.<br>Suite 1200<br>Washington, DC 20005-4051 |             |                      | 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.



### **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



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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

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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).

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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



UTILITY PATENT

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|                        |   |                               |
|------------------------|---|-------------------------------|
| 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**

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Dear Sir:

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**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 is 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.

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U.S. Patent Application No. 13/071,105

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    | <p>A method for detecting a genetic sequence in a mixed population of nucleic acid sequences, comprising:</p> <p>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;</p> <p>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;</p> | <p>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</p> <p>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....<br/>         Alternatively, dilute sources of template nucleic acids can be used. Page 7, paragraph 2</p> <p>Preferably at least ten diluted assay samples are amplified and analyzed. Page 10, paragraph 3</p> <p>Desirably each assay sample prior to amplification will contain less than a hundred or less than ten template molecules. Page 7, paragraph 2</p> <p>At its limit, single template molecules can be amplified so that the products are completely mutant or completely wild-type</p> |

| Claim | Claim recitations                                                                                                                                                       | Specification Support                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                   |
|-------|-------------------------------------------------------------------------------------------------------------------------------------------------------------------------|-----------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------|
|       | <p>determining nucleic acid sequence of amplification products from an assay sample with homogeneous amplification products.</p>                                        | <p>(WT). The homogeneity of these amplification products makes them trivial to distinguish through existing techniques. Page 6, last paragraph.</p> <p>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</p> <p>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</p> |
| 50    | <p>The method of claim 1 wherein each of the assay samples has on average 0.5 molecules of template.</p>                                                                | <p>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</p>                                                                                                                                                                                                                                                                                                                                                                                                                             |
| 51    | <p>The method of claim 1 wherein between 0.1 and 0.9 of the assay samples yield an amplification product</p>                                                            | <p>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</p>                                                                                                                                                                                                                                                                                                                                                                                  |
| 52    | <p>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.</p> | <p>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</p>                                                                                                                                                                                                                                                                                                                                                                                  |
| 53    | <p>The method of claim 1 wherein the mixed population of nucleic acid sequences is from a</p>                                                                           | <p>Biological samples which can be used as the starting material for the analyses may be from any</p>                                                                                                                                                                                                                                                                                                                                                                                                                                                                                   |

| 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.     | <p>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</p> <p>Desirably each assay sample prior to amplification will contain less than a hundred or less than ten template molecules. Page 7, paragraph 2</p> |
| 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.      | <p>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</p> <p>Desirably each assay sample prior to amplification will contain less than a hundred or less than ten template molecules. Page 7, paragraph 2</p> |
| 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. | <p>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</p> <p>Desirably each assay sample prior to amplification will</p>                                                                                      |

| 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<br><br>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<br><br>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<br><br>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                                                                                                                                                                                                                                                                                                                                                              |
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|       | five assay samples comprise less than ten template molecules.                                                                                                                                    | <p>100, 500, or 1000 diluted assay samples are amplified and analyzed. Page 10, paragraph 3</p> <p>Desirably each assay sample prior to amplification will contain less than a hundred or less than ten template molecules. Page 7, paragraph 2</p>                                                                                                                                |
| 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.  | <p>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</p> <p>Desirably each assay sample prior to amplification will contain less than a hundred or less than ten template molecules. Page 7, paragraph 2</p> |
| 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. | <p>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</p> <p>Desirably each assay sample prior to amplification will contain less than a hundred or less than ten template molecules. Page 7, paragraph 2</p> |
| 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. | <p>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</p> <p>Desirably each assay sample prior to amplification will contain less than a hundred or</p>                                                       |

| 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.       | <p>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</p> <p>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</p> |
| 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.              | <p>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</p> <p>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</p>                                            |
| 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. | <p>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</p> <p>To achieve a dilution to</p>                                                                                                                                                                        |



| 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. | <p>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</p> <p>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</p> |

Respectfully submitted,

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

Customer No. 11332

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