PTO/SB/57 (02-13)
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U.S. Patent and Trademark Office; U.S. DEPARTMENT OF COMMERCE

(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 2

Date: June 17, 2013

1. ☑ This is a request for *ex parte* reexamination pursuant to 37 CFR 1.510 of patent number 7,824,889 issued November 2, 2010. 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) 1-22 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.

13. [✔] The attached detailed request includes at least the following items:

a. A statement identifying each substantial new question of patentability based on prior patents and printed publications. 37 CFR 1.510(b)(1).

b. 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 6440706 & 7015015

[ ] 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/ — Authorized Signature

6/17/13 — Date

Ashita Doshi — Typed/Printed Name

57,327 — Registration No.

[ ] For Patent Owner Requester

[✔] For Third Party Requester

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re *Ex Parte* Reexamination of U.S. Patent No. 7,824,889	Examiner: To Be Assigned
Control No.: To Be Assigned	Art Unit: To Be Assigned
Reexam Filing Date: To Be Assigned	Confirmation No.: To Be Assigned
For: DIGITAL AMPLIFICATION	

REQUEST FOR *EX PARTE* REEXAMINATION UNDER 37 C.F.R. §1.510

Mail Stop *Ex Parte* Reexam
Commissioner for Patents
P.O. Box 1450
Alexandria, VA 22313-1450

Dear Sir:

On behalf of Life Technologies Corp. (hereinafter "Requester"), under provisions of 37 C.F.R. §1.510 *et seq.*, the undersigned hereby submits a Request for Reexamination of claims 1-22 of U.S. Patent No. 7,824,889 entitled "DIGITAL AMPLIFICATION" ("the '889 patent"). The '889 patent indicates on its face that it is assigned to The Johns Hopkins University.

Entry and consideration are respectfully requested.

Pursuant to 37 C.F.R §1.510, included with this Request are:

- the fee for requesting *ex parte* reexamination (37 C.F.R. §1.20(c)(1));
- an identification of the reexamined patent by patent number and every claim for which reexamination is requested;

- a citation of the patents and printed publications that are presented to provide a substantial new question of patentability, listed on form PTO/SB/08A;
- a statement identifying each substantial new question of patentability based on the cited patents and printed publications, and a detailed explanation of the pertinence and manner of applying the patents and printed publications to every claim for which reexamination is requested;
- a copy of every patent or printed publication relied upon or referred to in the Request;
- a copy of the entire patent including the front face, drawings, and specification/claims (in double-column format) for which reexamination is requested, and a copy of any disclaimer, certificate of correction, or reexamination certificate issued in the patent as Exhibit 1;
- a certification that the Request has been served in its entirety on the patent owner (through the attorney of record during prosecution) at the address shown in the accompanying Certificate of Service;
- a showing that the attorney filing this request has the authority to act on behalf of the real party in interest pursuant to 37 C.F.R. §1.34(a) under either a power of attorney from that party or in a representative capacity pursuant to §1.34.

TABLE OF CONTENTS

TABLE OF EXHIBITS

Patent for which *Inter Partes* Reexamination Is Requested

Exhibit 1: U.S. Pat. No. 7,824,889 to Vogelstein et al., titled "Digital Amplification," issued on November 2, 2010, with a priority date of August 2, 1999 and terminal disclaimer filed March 12, 2010.

Prior Art References Relied Upon for SNQs

Exhibit PA-1: Bischoff *et al.*, Hum Mol Genet. 4(3):395-9 (Mar 1995)

Exhibit PA-2: Kalinina *et al.*, Nuc. Acids Res. 25(10):1999-2004 (May 1997)

Exhibit PA-3: Zhang *et al.,* PNAS USA, 89(13):5847-51 (July 1, 1992),

Exhibit PA-4: Li *et al.*, Nature. 29;335(6189):414-7 (Sep 29, 1988)

Exhibit PA-5: Ruano *et al.*, Nucleic Acids Res. 17(20):8392 (Oct 25, 1989)

Additional Exhibits

Exhibit 2: PTO Form SB/08A

Exhibit 3: Relevant portions of prosecution history of U.S. Pat. No. 7,824,889

Exhibit 4: Relevant portions of prosecution history of U.S. Pat. No. 6,440,706

Exhibit 5: Lapidus et al., U.S. Pat No 5,928,870

Exhibit 6: Ruano et al., PNAS vol. 87 pp. 6296-6300, August 1990.

Exhibit 7: U.S. Pat. No. 7,915,015

Exhibit 8: Brenner *et al.*, Cancer Res. 55, 2892-2895 (July 1, 1995)

Exhibit 9: Cheung *et al.*, PNAS vol. 93 no. 25, pages 14676-14679 (Dec. 1996)

Exhibit 10: von Eggeling *et al.*, Hum. Genet. 99(2), pp 266-270 (Jan. 1997)

Exhibit 11: Prosecution history of continuing App. No. 13/071,105

I. IDENTIFICATION OF CLAIMS FOR WHICH REEXAMINATION IS REQUESTED AND BRIEF LISTING OF THE APPLIED ART, SUBSTANTIAL NEW QUESTIONS OF PATENTABILITY AND PROPOSED REJECTIONS

Ex parte reexamination is respectfully requested under 35 U.S.C. §§302-307 and 37 C.F.R. §1.510 of claims 1-22 of U.S. Patent No. 7,824,889 to Vogelstein *et al.* ("the '889 patent"), and currently assigned to The Johns Hopkins University. The '889 patent issued on November 2, 2010, with a priority date of August 2, 1999.

Reexamination of claims 1-22 is requested in view of one or more of the references applied herein. The SNQs listed in Table II are based on the applied references cited herein and summarized in Table I below. The proposed rejections for each SNQ are summarized in Table III below.

Table I: Summary of References Applied[1]				
Exh. No.	**Reference**	**Art Under:**	**Originally Cited?**	**Originally Relied On Or Discussed?**
PA-1	**"BISCHOFF"** Bischoff *et al.*, Hum Mol Genet. 4(3):395-9 (Mar 1995)	**102(B)/ 103**	**NO**	**NO**
PA-2	**"KALININA"** Kalinina *et al.*, Nucleic Acids Res. 25(10):1999-2004 (May 1997)	**102(B)/ 103**	**NO**	**NO**
PA-3	**"ZHANG"** Zhang *et al.*, PNAS USA, 89(13):5847-51 (July 1, 1992),	**102(B)/ 103**	**YES**	**NO**
PA-4	**"LI"** Li *et al.*, Nature. 29;335(6189):414-7 (Sep 29, 1988)	**102(B)/ 103**	**YES**	**NO**
PA-5	**"RUANO II"** Ruano *et al.*, Nucleic Acids Res. 17(20):8392 (Oct 25, 1989)	**102(B)/ 103**	**NO**	**NO**

Table II: Summary of SNQs	
SNQ No. 1:	Bischoff anticipates claims 1, 5, 8-15, 19, 20 & 22 under 35 U.S.C. § 102(b)
SNQ No. 2:	Claims 2-3 of the '889 patent are obvious under 35 U.S.C. § 103(a) over Bischoff in view of Kalinina
SNQ No. 3:	Claims 4, 6 & 7 of the '889 patent are obvious under 35 U.S.C. § 103(a) over Bischoff in view of Zhang
SNQ No. 4:	Claims 16, 17 & 20 of the '889 patent are obvious under 35 U.S.C. § 103(a) over Bischoff in view of Li
SNQ No. 5:	Claims 18, 20 & 21 of the '889 patent are obvious under 35 U.S.C. § 103(a) over Bischoff in view of Ruano II

[1] Applied references that are newly cited in this request are listed on the attached form SB/08A (Exhibit 2).

Table III Proposed Rejections	
Proposed Rejection No. 1:	Bischoff anticipates claims 1, 5, 8-15, 19, 20 & 22 under 35 U.S.C. § 102(b)
Proposed Rejection No. 2:	Claims 2-3 of the '889 patent are obvious under 35 U.S.C. § 103(a) over Bischoff in view of Kalinina
Proposed Rejection No. 3:	Claims 4, 6 & 7 of the '889 patent are obvious under 35 U.S.C. § 103(a) over Bischoff in view of Zhang
Proposed Rejection No. 4:	Claims 16, 17 & 20 of the '889 patent are obvious under 35 U.S.C. § 103(a) over Bischoff in view of Li
Proposed Rejection No. 5:	Claims 18, 20 & 21 of the '889 patent are obvious under 35 U.S.C. § 103(a) over Bischoff in view of Ruano II

II. CONCURRENT LITIGATION AND REEXAMINATION PROCEEDINGS: THE CLAIMS OF THE '889 PATENT ARE GIVEN THEIR BROADEST REASONABLE INTERPRETATION IN REEXAMINATION, UNLIKE THE STANDARDS APPLICABLE IN THE CONCURRENT LITIGATION

The '889 patent is presently involved in litigation in the United States District Court for the Middle District of North Carolina Greensboro Division (Esoterix Genetic Laboratories, LLC and The Johns Hopkins University vs. Life Technologies Corporation, Applied Biosystems, LLC, and Ion Torrent Systems, Inc., Case No. 12-1173 (filed October 31, 2012)).

The claims of the '889 Patent do not need to be "interpreted" in any particular manner to be found unpatentable over the prior art (*e.g.,* by their plain terms each of the limitations is found in the prior art). Nevertheless, claim interpretation in the reexamination process differs from that in other contexts, such as litigation in the federal courts. Therefore, Requester here summarizes the standards applicable in reexamination

and emphasizes that this Request addresses the claims using that claim interpretation standard, rather than the standards that are applicable outside the reexamination context.

In the context of reexamining patent claims, "the PTO must apply the broadest reasonable meaning to the claim language, taking into account any definitions presented in the specification." *In re Bass*, 314 F.3d 575, 577 (Fed. Cir. 2002) (citing *In re Yamamoto*, 740 F.2d 1569, 1571 (Fed. Cir. 1984)); *see also* 37 C.F.R. § 1.555(b). Giving claims their broadest reasonable construction "serves the public interest by reducing the possibility that claims, finally allowed, will be given broader scope than is justified." *In re Yamamoto*, 740 F.2d at 1571. "Construing claims broadly during prosecution is not unfair to the applicant (or, in this case, the patentee), because the applicant has the opportunity to amend the claims to obtain more precise claim coverage." *In re Am. Acad. of Sci. Tech Ctr.*, 367 F.3d 1359, 1363 (Fed. Cir. 2004) (citing *Yamamoto*, 740 F.2d at 1571-72).

While district courts interpret claim language in issued patents in light of the specification, prosecution history, prior art and other claims, this is not the mode of claim interpretation to be applied during examination, including reexamination. During examination, the claims must be interpreted as broadly as their terms reasonably allow. "The USPTO uses a different standard for construing claims than that used by district courts; during examination the USPTO must give claims their broadest reasonable interpretations." MPEP § 2111.01 (citing *Am. Acad. of Sci. Tech Ctr.,* 367 F.3d at 1363). The words of the claim must be given their plain meaning unless the applicant has provided a clear definition in the specification. *In re Zletz*, 893 F.2d 319, 321, 13 U.S.P.Q.2d 1320, 1322 (Fed. Cir. 1989). "[I]n proceedings before the PTO, claims in an

application are to be given their broadest reasonable interpretation consistent with the specification . . . as it would be interpreted by one of ordinary skill in the art." *In re Cortright*, 165 F.3d 1353, 1359 (Fed. Cir. 1999) (citing *In re Bond*, 910 F.2d 831, 833 (Fed. Cir. 1990)). Thus, in the analysis and discussion presented below, the identified claims are given their broadest reasonable interpretation.

Because the standards of claim interpretation used in the courts in patent litigation are different from the claim interpretation standards used in the Office in claim examination proceedings (including reexamination), any claim interpretations submitted herein for the purpose of demonstrating an SNQ are neither binding upon Requester in any litigation related to the '889 patent, nor do such claim interpretations necessarily correspond to the construction of claims under legal standards that are mandated to be used by the Courts in litigation. *See* 35 U.S.C. § 314; *see also* MPEP § 2686.04 II (determination of a SNQ is made independently of a Court's decision on validity because of different standards of proof and claim interpretation employed by the District Courts and the Office); *In re Trans Texas Holdings Corp.,* 498 F.3d 1290 (Fed. Cir. 2007), at 1297-98; *In re Zletz*, 893 F.2d at 322.

The interpretation and/or construction of the claims in the '889 patent presented either implicitly or explicitly herein should not be viewed as constituting, in whole or in part, Requester's own interpretation and/or construction of such claims, but instead should be viewed as constituting an interpretation and/or construction required by the standards applicable in the reexamination context and by Patent Owner's use of broad (and often expansive and undefined) terminology in the claims. Furthermore, Requester expressly reserves the right to present its own interpretation of such claims at a later time

during the related litigation, which interpretation may differ, in whole or in part, from that presented herein.

III. SUMMARY OF THE CLAIMS

U.S. Patent No. 7,824,889 (the '889 patent) is generally drawn to methods of determining allelic imbalance. The claims for which reexamination is requested read as follows:

> 1. A method for determining an allelic imbalance in 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 a selected genetic sequence on a first chromosome and a second number of assay samples which contain a reference genetic sequence on a second chromosome, wherein between 0.1 and 0.9 of the assay samples yield an amplification product;
>
> comparing the first number of assay samples to the second number of assay samples to ascertain an allelic imbalance in the biological sample.
>
> 2. The method of claim 1 wherein the step of amplifying employs real-time polymerase chain reactions.
>
> 3. The method of claim 2 wherein the real-time polymerase chain reactions comprise a dual-labeled fluorogenic probe.
>
> 4. The method of claim 1 wherein the selected genetic sequence and the reference genetic sequence are non-polymorphic markers.
>
> 5. The method of claim 1 wherein the biological sample is from blood.
>
> 6. The method of claim 1 wherein the selected genetic sequence is a non-polymorphic marker.

7. The method of claim 1 wherein the reference genetic sequence is a non-polymorphic marker.

8. The method of claim 1 wherein between 0.1 and 0.6 of the assay samples yield an amplification product.

9. The method of claim 1 wherein between 0.3 and 0.5 of the assay samples yield an amplification product.

10. The method of claim 1 wherein between 0.1 and 0.9 of the assay samples yield an amplification product as determined by amplification of the selected genetic sequence.

11. The method of claim 1 wherein between 0.1 and 0.9 of the assay samples yield an amplification product as determined by amplification of the reference genetic sequence.

12. The method of claim 1 wherein between 0.1 and 0.6 of the assay samples yield an amplification product as determined by amplification of the selected genetic sequence.

13. The method of claim 1 wherein between 0.1 and 0.6 of the assay samples yield an amplification product as determined by amplification of the reference genetic sequence.

14. The method of claim 1 wherein between 0.3 and 0.5 of the assay samples yield an amplification product as determined by amplification of the selected genetic sequence.

15. The method of claim 1 wherein between 0.3 and 0.5 of the assay samples yield an amplification product as determined by amplification of the reference genetic sequence.

16. The method of claim 1 wherein the set comprises at least 500 assay samples.

17. The method of claim 1 wherein the set comprises at least 1000 assay samples.

18. The method of claim 1 wherein the amplified molecules in each of the assay samples in the first and second numbers of assay samples are homogeneous such that the first number of assay samples do not contain the reference genetic sequence and the second number of assay samples do not contain the selected genetic sequence.

19. A method for determining an allelic imbalance in a biological sample, comprising the steps of:

distributing nucleic acid template molecules from 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 a selected genetic sequence on a first chromosome and a second number of assay samples which contain a reference genetic sequence on a second chromosome;

comparing the first number of assay samples to the second number of assay samples to ascertain an allelic imbalance between the first chromosome and the second chromosome in the biological sample.

20. The method of claim 19 wherein between 0.1 and 0.9 of the assay samples yield an amplification product.

21. The method of claim 20 wherein between 0.1 and 0.9 of the assay samples yield a homogeneous amplification product.

22. The method of claim 19 wherein the biological sample is blood.

IV. PROSECUTION HISTORY OF THE '889 AND PARENT '706 PATENT

During prosecution of the '889 patent, no prior art was applied against the '889 claims (except for the claims of the parent patent No. 6,440,706 in a double-patenting rejection).[2] The references provided and addressed in this reexamination request present substantial new questions of patentability because, among other things, they teach one or more elements of the '889 claims, and either anticipate or render these claims obvious.

Although no art was applied against the '889 claims, during the prosecution of the parent patent (U.S. 6,440,706, hereafter the '706 patent, for which Requester is concurrently requesting reexamination) art was applied to the claims. For the purposes of

[2] Prosecution history of the '889 patent, Office Action mailed Dec. 29, 2009, at page 2 (Exhibit 3).

patentability in this reexamination, the '706 claims were substantially similar to the '889 claims. Generally speaking, claims of both the '706 and '889 patents recite a method requiring four steps: (1) forming a set of assay samples containing template molecules from a biological sample (*e.g.*, by "distributing"); (2) amplifying the template molecules in the assay samples; (3) analyzing the amplified molecules to determine a first number of assay samples that contains one sequence and a second number of assay samples that contains a different sequence; and (4) comparing the numbers of assay samples. The '706 claims generally require that the last comparing step is performed to ascertain a ratio that reflects the composition of the biological sample, whereas the '889 claims generally require that the comparing is performed to ascertain an allelic imbalance.

During original prosecution of the '706 claims, the PTO rejected the '706 claims as obvious over a reference by Lapidus et al.[3] in view of a publication by Ruano ("Ruano I").[4] In particular, the PTO found that Lapidus taught all steps of '706 claims except for an initial set/forming/diluting step, whereas Ruano I taught single-molecule dilution, and it would have been obvious to combine Lapidus and Ruano I to arrive at the claimed method.[5] In response, the '706 applicants argued that neither Lapidus nor Ruano I counted numbers of assay samples. In particular, the applicants argued that:

> 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

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[3] Lapidus et al.. U.S. Pat No 5,928,870 (Exhibit 5).

[4] Ruano et al., PNAS vol. 87 pp. 6296-6300, August 1990 (Exhibit 6). A different publication by Ruano et al., (Ruano II) is being applied as a secondary reference in this request.

[5] '706 patent prosecution history, Office Action issued April 12, 2001, at page 6 (Exhibit 4)

> assay samples are not determined according to Lapidus, neither are the numbers compared, as required in step 4.[6]

The PTO ultimately allowed the claims on the grounds that the closest prior art (Lapidus) taught amplification and concentration determination of a reference and target nucleic acid, but that Lapidus' "determination of concentration is within a sample"[7] and … did not teach or suggest forming a set of assay samples by dilution.

The references applied in this reexamination request teach the elements that the '706 applicants asserted were missing from the prior art (*i.e.*, forming a set of a plurality of assay samples, for example by dilution). In contrast to Lapidus, the primary references and most of the secondary references applied herein do teach determining a number of assay samples.

V. SUBSTANTIAL NEW QUESTIONS OF PATENTABILITY

This section demonstrates how the applied prior art references, either alone or in combination raise substantial new questions ("SNQs") of patentability with respect to each claim of the '889 patent for which reexamination is sought. *Ex parte* reexamination of claims 1-22 of the '889 patent is respectfully requested. These references were either not of record and/or not considered by the Examiner. These references raise substantial new questions ("SNQs") of patentability and render the claims unpatentable. A brief statement of the SNQs of patentability is set forth immediately below. A detailed explanation of the pertinence and manner of applying the cited prior art to each claim for which reexamination is sought is presented in **Section VI** below.

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[6] '706 patent prosecution history, Amendment dated July 12, 2001, at page 12 (Exhibit 4).

[7] '706 patent prosecution history, Supplemental Notice of Allowability mailed March 26, 2002, at page 2 (Exhibit 4).

A. SNQ No. 1: Bischoff anticipates claims 1, 5, 8-15, 19, 20 & 22 under 35 U.S.C. § 102(b)

Bischoff[8] was published in March 1995 and is thus prior art to the '889 patent under 35 U.S.C. § 102(b). Bischoff is newly cited in the present request. Under the broadest reasonable interpretation of the claims, Bischoff discloses methods that meet all of the limitations of the methods of claims 1, 5, 8-15, 19, 20 & 22.

SNQ No. 1 based on Bischoff is new for at least two reasons: (i) Bischoff is newly cited in the present request and was not before the PTO during original prosecution; and (ii) the explanation presented herein of how Bischoff anticipates various claims presented herein was not before the original Examiner.

SNQ No. 1 based on Bischoff is substantial at least because Bischoff teaches all aspects of claims 1, 5, 8-15, 19, 20 & 22 and squarely anticipates these claims. In contrast, during the original prosecution of the '889 patent no art was found to anticipate the claims.

Thus, a substantial new question of patentability based on Bischoff alone is raised with respect to claims 1, 5, 8-15, 19, 20 & 22.

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[8] Bischoff *et al.*, *Single cell analysis demonstrating somatic mosaicism involving 11p in a patient with paternal isodisomy and Beckwith-Wiedemann syndrome.* Hum Mol Genet. 4(3):395-9 (Mar 1995), which forms prior art to the '889 patent under 35 U.S.C. § 102(b) (Exhibit PA-1).

B. SNQ No. 2: Claims 2-3 of the '889 patent are obvious under 35 U.S.C. § 103(a) over Bischoff in view of Kalinina

Bischoff has been discussed above in SNQ No. 1. Kalinina[9] was published on May 15, 1997 and is prior art to the '889 patent under 35 U.S.C. § 102(b). Kalinina is newly cited in the present request.

Bischoff and Kalinina together raise a new question of patentability as to claims 2 and 3 because they were neither cited nor considered during the prosecution of the '889 patent or its parent '706 patent.

Bischoff and Kalinina together raise a substantial question of patentability because it would have been obvious to those of ordinary skill in the art to practice the methods of claims 2 and 3 in light of the combined teachings of Bischoff and Kalinina. Exemplary rationales as to why Bischoff's and Kalinina's combined teachings would have rendered the claims obvious are presented in more detail in the next section applying the art to the claims.

Thus, a substantial new question of patentability based on Bischoff and Kalinina is raised with respect to claims 2 and 3.

C. SNQ No. 3: Claims 4, 6 and 7 of the '889 patent are obvious under 35 U.S.C. § 103(a) over Bischoff in view of Zhang

Bischoff has been discussed above in SNQ No. 1. Zhang[10] was published on July 1, 1992 and is prior art to the '889 patent under 35 U.S.C. § 102(b).

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[9] Kalinina *et al.*, *Nanoliter scale PCR with TaqMan detection*. Nucleic Acids Res. 25(10):1999-2004 (May 15, 1997), forming prior art to the '889 patent under 35 U.S.C. § 102(b) (Exhibit PA-2).

Bischoff and Zhang together raise a new question of patentability as to claims 4, 6 & 7 at least because Bischoff was neither cited nor considered during the prosecution of the '889 patent. Zhang was not discussed or relied on during original prosecution although it was cited by the applicants.

Bischoff and Zhang together raise a substantial question of patentability because it would have been obvious to those of ordinary skill in the art to practice the methods of claims 4, 6, and 7 in light of the combined teachings of Bischoff and Zhang. Exemplary rationales as to why Bischoff's and Zhang's combined teachings would have rendered the claims obvious are presented in more detail in the next section applying the art to the claims.

Thus, a substantial new question of patentability based on Bischoff and Zhang is raised with respect to claims 4, 6 and 7.

D. SNQ No. 4: Claims 16, 17 and 20 of the '889 patent are obvious under 35 U.S.C. § 103(a) over Bischoff in view of Li

Bischoff has been discussed above in SNQ No. 1. Li[11] was published on September 29, 1988 and is prior art to the '889 patent under 35 U.S.C. § 102(b). Although cited by the applicants, Li was not discussed or relied on during original prosecution. In addition, Li has been cited against a related continuing application No. 13/071,105, as anticipating the pending claims, indicating that it is highly likely that the

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[10] Zhang *et al., Whole genome amplification from a single cell: implications for genetic analysis*. PNAS USA, 89(13):5847-51 (July 1, 1992), forming prior art under 35 U.S.C. § 102(b) to the '889 patent (Exhibit PA-3).

[11] Li *et al., Amplification and analysis of DNA sequences in single human sperm and diploid cells.* Nature. 29;335(6189):414-7 (Sep 29, 1988), which forms prior art to the '889 patent under 35 U.S.C. § 102(b) (Exhibit PA-4).

examiner would also have rejected the claims of the' 889 patent, which are similar to the rejected claims of the '105 application.

Bischoff and Li together raise a new question of patentability as to claims 16, 17 and 20 at least because Bischoff was neither cited nor considered during the prosecution of the '889 patent. Also, Li was not specifically considered during original prosecution although it was cited by the applicants.

Bischoff and Li together raise a substantial question of patentability because it would have been obvious to those of ordinary skill in the art to practice the methods of claims 16, 17 and 20 in light of the combined teachings of Bischoff and Li. Exemplary rationales as to why Bischoff's and Li's combined teachings would have rendered the claims obvious are presented in more detail in the next section applying the art to the claims.

Thus, a substantial new question of patentability based on Bischoff and Li is raised with respect to claims 16, 17 and 20.

E. SNQ No. 5: Claims 18, 20 and 21 of the '889 patent are obvious under 35 U.S.C. § 103(a) over Bischoff in view of Ruano II

Bischoff has been discussed above in SNQ No. 1. Ruano II[12] was published on October 25, 1989 and is prior art to the '889 patent under 35 U.S.C. § 102(b). Ruano II is newly cited in the present request.

[12] Ruano *et al.*, Nucleic Acids Res. 17(20):8392 (Oct 25, 1989), which forms prior art to the '889 patent under 35 U.S.C. § 102(b) (Exhibit PA-5).

Bischoff and Ruano II together raise a new question of patentability as to claims 18, 20 and 21 because they were neither cited nor considered during the prosecution of the '889 patent.

Bischoff and Ruano II raise a substantial question of patentability because it would have been obvious to those of ordinary skill in the art to practice the methods of claims 18, 20 & 21 in light of the combined teachings of Bischoff and Ruano II. Exemplary rationales as to why Bischoff's and Ruano II's combined teachings would have rendered the claims obvious are presented in more detail in the next section applying the art to the claims.

Thus, a substantial new question of patentability based on Bischoff and Ruano II is raised with respect to claims 18, 20 and 21.

VI. MANNER OF APPLYING THE CITED PRIOR ART AND PROPOSED REJECTIONS

A. Proposed rejection 1: Bischoff anticipates claims 1, 5, 8-15, 19, 20 and 22 under 35 U.S.C. § 102(b)

1. Short introductory overview of relevant portions of Bischoff's disclosure

Independent claim 1 is anticipated by Bischoff.[13] To provide a quick orientation to the Examiner, this section presents an **introductory high-level overview** of the steps of the claims and broadly maps Bischoff's experiments onto each of these steps. **A more detailed application of Bischoff's teachings to each claimed step, showing the details**

[13] Bischoff *et al.*, *Single cell analysis demonstrating somatic mosaicism involving 11p in a patient with paternal isodisomy and Beckwith-Wiedemann syndrome.* Hum Mol Genet. 4(3):395-9 (Mar 1995), which forms prior art to the '889 patent under 35 U.S.C. § 102(b) (Exhibit PA-1).

of how Bischoff performed each step with specific cites to Bischoff's relevant disclosure is presented in the next section.

Generally, the independent method claims (claim 1 and 19) of the '889 patent recite four steps: (1) distributing template molecules from a biological sample to form a set of assay samples (recited in claim 19 but not claim 1); (2) amplifying the template molecules within the assay samples; (3) analyzing the amplified molecules to determine a first number of assay samples that contain a "*selected genetic sequence*" and a second number of assay samples that contain a "*reference genetic sequence*;" and (4) comparing the two numbers of assay samples to ascertain an allelic imbalance in the biological sample.

Bischoff anticipates both independent claims of the '889 patent and many of the dependent claims as well. Bischoff suspected that part of the 11p arm on maternal chromosome 11 was lost in a subset of cells in a patient suffering from Beckwith-Wiedemann syndrome. To resolve this question, Bischoff performed all steps of independent claims 1 and 19 of the '889 patent, as follows.

- ***Distributing and/or set-forming step (recited in claim 19 but not claim 1)***
  - This step involves "*distributing template molecules from a biological sample to form a set comprising a plurality of assay samples.*"
  - Bischoff started by isolating six single cells (lymphocytes) from a peripheral blood sample of his patient into separate reaction tubes. Each lymphocyte cell contained template molecules from a biological sample.

- ***Amplifying step***
  - This step involves "*amplifying the template molecules within the assay samples to form a population of amplified molecules in the assay samples of the set.*"

- Bischoff subjected each of his six single-cell assay samples to a random-primed whole-genome amplification reaction that amplified the template molecules in the single cells (a "PEP" reaction, explained in the next section).

❖ ***Analyzing/determining step***

- This step involves "*analyzing the amplified molecules ... to determine a first number of assay samples which contain a selected genetic sequence on a first chromosome and a second number of assay samples which contain a reference genetic sequence on a second chromosome.*"

- Bischoff analyzed the PEP amplification products from each sample to determine whether each parental allele at four different marker loci was present,[14] using four separate secondary locus-specific PCR reactions and gel electrophoresis.

- Bischoff counted the number of single-cell samples containing a first allele of interest, thereby "*determining a first number of assay samples which contain a selected genetic sequence on a first chromosome.*"

- Bischoff also counted the number of samples containing a second allele of interest, thereby "*determining a second number of assay samples which contain a reference genetic sequence on a second chromosome.*"

- Bischoff chose various different combinations of alleles as the "*selected*" and "*reference genetic sequence,*" as described further in the "comparing" step below.

❖ ***Comparing step***

- This step involves "*comparing the first number of assay samples to the second number of assay samples to ascertain an allelic imbalance ... in the biological sample,*" where claim 19 also specifies that the allelic imbalance is "*between the first chromosome and the second chromosome*"

- Bischoff suspected that part of the "p" arm on maternal chromosome 11 was lost in a subset of cells. To resolve this question, Bischoff made various comparisons each using different combinations of "*selected*" and "*reference*" sequence:

[14] Specifically, Bischoff examined two loci on the 11p arm of chromosome 11 (*i.e.*, the HBB and D11S904 loci), one locus on the opposite arm 11q of chromosome 11 (*i.e.*, the CD3D locus), and one locus on chromosome 21 (the INFAR locus).

- **Comparison 1 (between two non-homologous chromosomes, chromosomes 11 and 21)** A comparison between maternal alleles on two non-homologous maternal chromosomes 11 and 21 as follows:

  - Bischoff compared the number of assay samples containing:
    - a "*selected genetic sequence on a first chromosome*" in the form of a maternal allele at a locus on the "p" arm of chromosome 11
    - a "*reference genetic sequence on a second chromosome*" in the form of the maternal allele at a locus on chromosome 21.
  - Bischoff compared two distinct 11p loci (HBB and D11S904) to a single locus on chromosome 21 (INFAR).

- **Comparison 2 ("intra-locus," two homologous alleles on the suspect "p" arm of chromosome pair 11)** Bischoff suspected that part of the 11p arm was lost on maternal chromosome 11. To ascertain if that were so, he compared the number of cells containing a maternal allele on the suspect "p" arm of maternal chromosome 11 with the number of cells containing the corresponding paternal allele on paternal chromosome 11.

  - Bischoff compared the number of assay samples containing:
    - a "*selected genetic sequence on a first chromosome*" in the form of a maternal allele at an 11p locus (*i.e.*, a locus on the "p" arm of maternal chromosome 11), and
    - a "*reference genetic sequence on a second chromosome*" in the form of the paternal allele at the same 11p locus on paternal chromosome 11.
  - Bischoff made this comparison at each of two distinct 11p loci (HBB and D11S904).

- **Comparison 3 ("intra-locus," two homologous alleles on the non-suspect "q" arm of chromosome pair 11)** Bischoff compared the number of cells containing a maternal allele on the non-suspect "q" arm of maternal chromosome 11 with the number of cells containing the corresponding paternal allele on paternal chromosome 11.

  - Bischoff compared the number of assay samples containing:
    - a "*selected genetic sequence on a first chromosome*" in the form of one maternal allele at the 11q locus CD3D (*i.e.*, a locus on the non-suspect "q" arm of maternal chromosome 11), and
    - a "*reference genetic sequence on a second chromosome*" in the form of the paternal CD3D allele on paternal chromosome 11.

- **Comparison 4 ("intra-locus," two homologous alleles on non-suspect chromosome pair 21)** Bischoff compared the number of cells containing a maternal allele on maternal chromosome 21 with the number of cells containing the corresponding paternal allele on paternal chromosome 21.

  - Bischoff compared the number of assay samples containing:
    - a "*selected genetic sequence on a first chromosome*" in the form of one maternal allele at the locus INFAR on maternal chromosome 21 and
    - a "*reference genetic sequence on a second chromosome*" in the form of the paternal INFAR allele on paternal chromosome 21.

2. Detailed explanation of the pertinency and manner of applying Bischoff to independent claim 1

This section discusses in more particular detail how, under the broadest reasonable interpretation of the claims, Bischoff discloses methods that meet each and every limitation of independent claim 1.

i) Bischoff discloses "A method for determining an allelic imbalance in a biological sample"

This language forms the preamble of claim 1. Under the PTO's standards for patentability, as opposed to validity, a preamble which merely recites an "intended use" does not limit the claim in any way.[15]

But even if the preamble were limiting (which it is not), Bischoff discloses "*determining an allelic imbalance in a biological sample*" under the broadest reasonable interpretation. The '889 patent does not expressly define "allelic imbalance," beyond giving one example of a PCR application in which allelic imbalance using "[q]uantitative

[15] *Rowe v. Dror*, 112 F.3d 473, 478, 42 USPQ2d 1550, 1553 (Fed. Cir. 1997) (preamble's recitation of an intended use is not a limitation).

analysis with non-polymorphic markers" using two probes that recognize sequences from different chromosomes is used. *See* Col. 5: 63-65. However, the claims of a related patent No. 7,915,015 which was filed as a continuation of the '889 patent makes clear that allelic imbalance is not restricted to non-polymorphic markers. In particular, claim 1 of the '015 patent is directed to a method of determining allelic imbalance by comparing the number of assay samples containing a first allelic form and a second allelic form of a marker.[16] Because the '015 patent and the '889 patent share the same specification, under the broadest reasonable interpretation "allelic imbalance" must also encompass imbalances between different (*e.g.*, polymorphic) allelic forms of a single marker. Requester will therefore proceed on the premise that assay samples in which at least a subset of diploid cells have lost one of a pair of two alleles have an "allelic imbalance" under the broadest reasonable interpretation.[17]

Bischoff "*determin*[*ed*] *an allelic imbalance*" as recited in claim 1, in the form of a loss of an allele in a subset of cells in a sample. Bischoff analyzed a patient with Beckwith-Wiedemann ("BWS") syndrome to determine the genetic event underlying the patient's condition.[18] Bischoff noted that some BWS patients had an allelic imbalance in

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[16] U.S. Pat. No. 7,915,015, claim 1, reciting a " for determining an allelic imbalance … comprising … determin[ing] first number of assay samples which contain a first allelic form of a marker and a second number of assay samples which contain a second allelic form of the marker," and comparing the two numbers. (Exhibit 7).

[17] *See, e.g.*, Brenner *et al.*, *Chromosome 9p Allelic Loss and p16/CDKN2 in Breast Cancer and Evidence of pl6 Inactivation in Immortal Breast Epithelial Cells.* Cancer Res. 55, 2892-2895 (July 1, 1995) (Exhibit 8), studying "allelic loss," (Title), also called "loss of heterozygosity" (Abstract) in cancer samples, and referring to "partial LOH" (*i.e.*, partial loss of heterozygosity in a subset of cells) as "allelic imbalance" at page 2892, left col., Section on "Microsatellite Length Polymorphism" (noting that "partial LOH and allelic imbalance were considered significant only if the signal intensity of one allele was diminished by approximately one-half or more of its normal intensity in relation to the remaining allele").

[18] Bischoff, Abstract.

the form of "partial paternal isodisomy of 11p"[19] Partial paternal 11p isodisomy (which Bischoff also referred to as 'uniparental disomy' or UPD) is a condition involving an aberrant unbalanced translocation of a chromosomal 11p region with subsequent retention of two copies of the paternal 11p region. In addition, the isodisomy in Bischoff's particular patient also "involve[ed] loss of the maternal 11p region in some cells,"[20] Thus, in some BWS patients, both chromosomes 11 carry the same 'isodisomic' 11p region, and within this 11p region each chromosome carries a copy of an allele inherited from the father (referred to as the "paternal allele" herein), and neither chromosome carries a copy of a corresponding allele inherited from the mother ("maternal allele"). Bischoff diagrams how this uniparental inheritance occurs in Figure 3, reproduced below.

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[19] Bischoff, Abstract.

[20] Bischoff, page 398, left col., top paragraph. *See also* Fig. 3. depicting paternal isodisomy of the 11p arm ("PAT UPD 11p") as a recombinant chromosome pair 11, where both homologous chromosomes have a "p" arm derived from the father, and neither has a "p" arm derived from the mother.

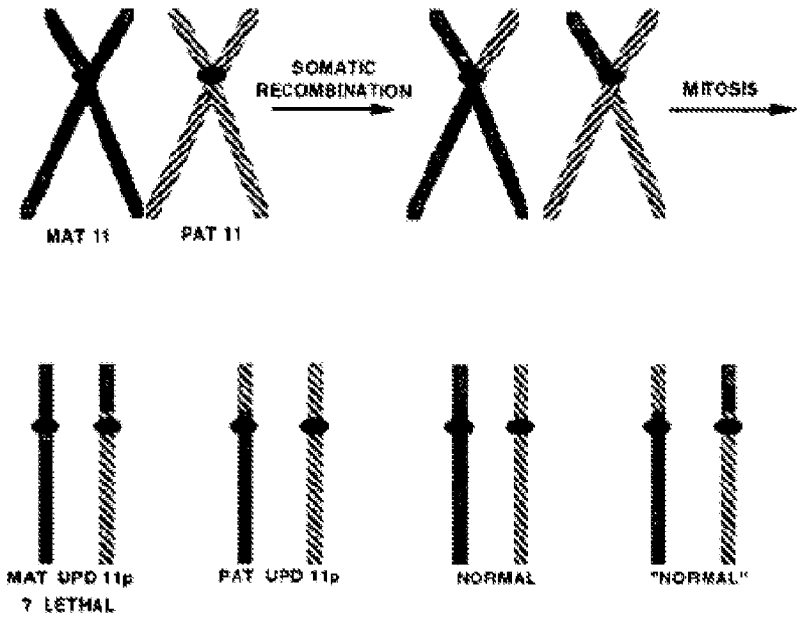

Figure 3. Diagram showing somatic recombination resulting in mosaicism for paternal isodisomy of 11p. Note that the recombination would lead to a population of cells with maternal isodisomy that is presumably lethal and a population of cells that are apparently normal by molecular analysis but contain both recombinant chromosomes.

For convenience, the chromosome that harbors maternal alleles in all genomic regions outside 11p shall be designated the maternal chromosome, and the other chromosome that is entirely paternal in origin shall be designated the paternal chromosome. Within the isodisomic portion of the 11p region, both the paternal and maternal chromosomes of chromosome pair 11 each carry a copy of the paternal allele, and neither carries a copy of the maternal allele (*see* bottom of Figure 3 above, the "PAT" and "UPD 11p" chromosomes).

It should be noted that Bischoff 's paternal isodisomy involved two separate and distinct genetic aspects: first, loss of maternal 11p alleles and second, disomy (two copies of paternal 11p alleles). **Only the first genetic aspect of isodisomy (loss of**

maternal 11p alleles) is relevant to this request – as explained below, Bischoff checked for maternal allelic loss using the claimed methods.[21]

Because Bischoff's paternal isodisomy involved a loss of maternal alleles that were originally present on the "p" arm of chromosome 11, paternal isodisomy is an "allelic imbalance" under the broadest reasonable interpretation. Bischoff concluded that the isodisomy was present in only a subset of cells, because he observed "somatic mosaicism"[22] in which some cells in the patient sample were isodisomic and had lost maternal alleles within the 11p region, but other cells in the sample were genetically normal and showed "normal biparental inheritance"[23] of both the maternal and paternal alleles in the 11p region. In particular, "[t]wo populations of cells were detected, a population of cells with normal biparental inheritance for chromosome 11 and a population of cells with partial paternal isodisomy of 11p."[24]

Bischoff also determined an allelic imbalance "*in a biological sample.*" In particular, Bischoff analyzed a "blood sample" from his patient,[25] which is explicitly recognized as a preferred biological sample in the '889 patent.[26]

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[21] Although Bischoff also checked for disomy of paternal alleles, this analysis is not relevant to this request. By way of explanation, Bischoff determined disomy by karyotype analysis, which revealed that all cells appeared to carry two intact chromosomes 11, both appearing to have an intact 11p arm ("High-resolution chromosome analysis revealed a normal 46, XY karyotype.") Because single-cell PCR had indicated indicated that some cells were apparently missing maternal alleles within the 11p arm of maternal chromosome 11 (at loci D11S904 and HBB), Bischoff therefore inferred that in these cells, the 11p arm found to be present on the maternal chromosome 11 by karyotype analysis must have been derived from the paternal chromosome, and that the patient thus had two 11p regions both derived from the father.

[22] Bischoff, Abstract

[23] Bischoff, Abstract

[24] Bischoff, Abstract.

[25] Bischoff, page 396, right col., last paragraph.

[26] '889 patent, col. 6, lines 57-62.

Because an allelic loss in a subset of blood cells in a sample is an "allelic imbalance in a biological sample" under the broadest reasonable interpretation, Bischoff's identification of partial paternal isodisomy is an "allelic imbalance." Accordingly, Bischoff "*determin*[*ed*] *an allelic imbalance in a biological sample*" under the broadest reasonable interpretation.

ii) *Bischoff discloses "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"*

Under the broadest reasonable meaning, Bischoff discloses the amplifying step recited in claim 1.

Bischoff started with "*template molecules ... obtained from a biological sample*" as recited in claim 1. First, Bischoff took a "blood sample" from his patient,[27] which is explicitly recognized as a "preferred" biological sample in the '889 patent.[28]

From this biological sample, Bischoff generated "*a set comprising a plurality of assay samples*" containing the "*template molecules*" by isolating "single blood lymphocytes"[29] to generate single-cell assay samples where each cell contains genomic template molecules. In particular, Bischoff explained that "[p]eripheral blood lymphocytes (uncultured) from the patient with BWS were individually visualized … and micromanipulated first into a wash droplet of DNA-free growth

[27] Bischoff, page 396, right col., last paragraph.
[28] '889 patent, col. 6, lines 57-62.
[29] Bischoff, page 396, right col., last paragraph.

media and then placed into separate reaction tubes."[30] Thus, each reaction tube contained a single-cell assay sample containing genomic template. Bischoff made and analyzed a set of six single-cell assay samples.[31]

Using the genome of each isolated cell as a source of template molecules, Bischoff next performed "[w]hole genome amplification … on [his] single cell [assay sample]s,"[32] in the form of a primer extension preamplification (PEP) reaction of the whole genome."[33] Under the broadest reasonable interpretation, the PEP amplification procedure resulted in "*amplifying template molecules*" by generating randomly-amplified fragments of the original genomic template molecules. Bischoff explained that his PEP procedure was a form of "[w]hole genome amplification"[34] which "allows for amplification of very small amounts of genetic material"[35] present in single-cell samples. Bischoff used the PEP amplification procedure developed by Zhang et al., which Zhang explained was an amplification method.[36] In addition, the art recognized PEP as an amplification reaction used to "amplify" genomic DNA, and more specifically recognized PEP as a type of "random PCR."[37] Thus, under the

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[30] Bischoff, page 398, right col., section titled "Single cell micromanipulation and PEP."

[31] *See, e.g.*, Bischoff, Table 1 (showing analysis of six individual cells)

[32] Bischoff, Abstract.

[33] Bischoff, sentence bridging pages 396-397.

[34] Bischoff, Abstract.

[35] Bischoff, page 397, sentence bridging left and right cols.

[36] Zhang *et al., Whole genome amplification from a single cell: implications for genetic analysis*. PNAS USA, 89(13):5847-51 (1992) (Exhibit PA-3), forming prior art under 35 U.S.C. § 102(b) to the '889 patent. Zhang is used herein as a secondary reference in some proposed rejections, and is also used as a primary reference in the concurrently-requested reexamination of related patent No 6,440,706.

[37] *See, e.g.,* Cheung *et al.*, PNAS vol. 93 no. 25, pages 14676-14679 (Dec. 1996) (Exhibit 9), at page 14676, left col., first paragraph (explaining that PEP "uses a random 15-mer to prime Taq DNA synthesis frequently throughout the genome, [and] has been used to ***amplify*** genomic DNA from as little as a single haploid cell and demonstrates good coverage"); *see also* von Eggeling *et al.*, Human Genetics Volume 99, Issue 2, pp 266-270, (Jan. 1997) (Exhibit 10), at

broadest reasonable interpretation, Bischoff "*amplif*[*ied*] *template molecules within a set comprising a plurality of assay samples*" as recited in claim 1.

Finally, Bischoff "*form*[*ed*] *a population of amplified molecules in each of the assay samples of the set*." In particular, Bischoff analyzed the PEP amplification products to check for the presence of each allele at four loci of interest. As shown in Table 2,[38] Bischoff successfully detected PEP amplification products of each locus, thereby showing that PEP amplification indeed had successfully generated "*a population of amplified molecules*" in each assay sample.

Accordingly, Bischoff discloses "*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*."

iii) Bischoff discloses "analyzing the amplified molecules in the assay samples of the set"

Under the broadest reasonable meaning of the claim terms, Bischoff performed a step of "*analyzing the amplified molecules in the assay samples of the set*" as recited in claim 1.

Under the broadest reasonable interpretation, "*analyzing the amplified molecules in the assay samples of the set*" encompasses analysis of aliquots of the amplified molecules by a secondary amplification reaction and electrophoresis.

Abstract (explaining that DNA of single cells "was ***amplified*** by at least 50-fold with a random-PCR technique, viz., primer extension preamplification").

[38] Bischoff, Table 2, page 397.

The '889 patent does not expressly define "analyzing the amplified molecules in the assay samples of the set." Instead, the '889 broadly allows any analytical method of choice, stating that "[a]lthough 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 using other types of probes, including TaqMan™ (dual-labeled fluorogenic) probes. . . , pyrene-labled probes, and other biochemical assays."[39] Hence, the broadest reasonable interpretation of "analysis" would encompass analysis by means of a secondary marker-specific PCR amplification followed by analysis of amplification products by gel electrophoresis, as Bischoff did.

In particular, after amplifying his template molecules by whole-genome PEP amplification, Bischoff analyzed the "resultant product" of PEP amplification by "locus specific microsatellite marker analysis" using a secondary PCR reaction.[40] The purpose of the locus-specific analysis was "to determine the [parental] chromosome 11 origins"[41] of alleles within the suspect 11p region. This locus-specific analysis included a "post-PEP PCR" with radiolabeled locus-specific primers, and followed by detection of the radiolabeled-amplified allelic products at each locus by gel electrophoresis and autoradiography.[42] Two 11p "markers HBB and D11S904 were selected for analysis …

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[39] '889 patent, Col. 7, lines 30-37,

[40] Bischoff, Abstract, *see also* page 385, last paragraph of Introduction (Locus specific amplification was performed to determine the chromosome 11 origins in the preamplified [PEP products of] individual cells").

[41] Bischoff, page 395, Introduction, last paragraph.

[42] Bischoff, page 398, section titled "Molecular analysis of genomic DNA" ("One primer from each set was end-labeled and used in PCR" after which "alleles were separated on a 6% denaturing polyacrylamide DNA sequencing gel at 70 W for 2-3 h. Gels were wrapped in plastic

[as well as two other] informative markers located outside of the BWS region, CD3D on 11q23 and INFAR on chromosome 21."[43] Figure 2 in Bischoff shows the "Post-PEP PCR of single cells" in which the single cell results are in lanes 4-9 of the depicted autoradiograph.[44]

Accordingly, Bischoff analyzed his PEP amplification products (*i.e.*, "*the amplified molecules in the assay samples*") by subjecting aliquots of his PEP amplification products to locus-specific analysis involving secondary amplification by PCR, gel electrophoresis, and autoradiography where such locus-specific analysis was informative of the PEP-amplified copies of each allele ("*amplified molecules*").. Under the broadest reasonable interpretation, as explained above, analyzing can include a multi-step process where one of the steps is a secondary amplification reaction.[45]

Therefore, Bischoff discloses "*analyzing the amplified molecules in the assay samples of the set*."

*iv) Bischoff's analysis involved both "**determin[ing]** a first number of assay samples which contain a selected genetic sequence on a first chromosome and a second number of assay samples which contain a reference genetic sequence on a second chromosome" and "**comparing** the first number ... to the second number of assay samples to ascertain an allelic imbalance"*

Bischoff analyzed his samples in several ways, each of which "***determine[s]*** *a first number of assay samples which contain a selected genetic sequence on a first*

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and exposed to Kodak XAR film for 2-16 h at - 80°C"); *see also* following section titled "Post-PEP PCR ("Each specific locus was amplified [and analyzed] as described above"), and Fig. 2.

[43] Bischoff, paragraph bridging pages 397-398.

[44] Bischoff, page 396.

[45] '889 patent, col. 7, lines 30-37. ("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 … [and] other biochemical assays.")

chromosome and a second number of assay samples which contain a reference genetic sequence on a second chromosome" and "***compar*[*ed*]** *the first number … to the second number to ascertain an allelic imbalance*" as recited in claim 1.

The '889 patent does not provide an explicit definition of "*selected genetic sequence on a first chromosome*" or *reference genetic sequence on a second chromosome*". Applicant used one molecular beacon probe to detect the presence of mutations in c-Ki-Ras at codons 12 and/or 13 and a separate molecular beacon probe to target a portion of the c-Ki-Ras gene that is not known to be mutated in cancers.[46] Under the broadest reasonable interpretation, a "*selected genetic sequence*" can be any first allelic sequence which Bischoff used for comparison, whereas a "*reference genetic sequence*" can be any second allelic sequence. This limitation also requires that the "selected genetic sequence" and the "reference sequence" are on a first and second chromosome, respectively. Because the specification of the '889 patent is silent as to what it means to be on a first and second chromosome, the plain meaning prevails under the broadest reasonable interpretation.[47] Under this interpretation, the maternal and paternal alleles at a single locus can be used as a "*selected*" and "*reference*" sequence respectively, since the maternal and paternal chromosomes bearing this locus are two different—albeit homologous--chromosomes. This interpretation is supported in independent claim 19, which relates to determining an allelic imbalance "between" a first chromosome carrying a first allelic form of a marker and a second chromosome carrying a second allelic form of the marker. Because two different sequences on non-

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[46] '889 patent, col .10, lines 16-54.
[47] *In re Bass*, 314 F.3d 575, 577 (Fed. Cir. 2002).

homologous chromosomes are not normally alleles of each other, "allelic imbalance" is normally between two homologous chromosomes bearing sister alleles.

Bischoff suspected that his blood sample contained an allelic imbalance in which a subset of cells in his biological sample had lost the "p" arm of maternal chromosome 11. To confirm his theory, Bischoff checked each of his single-cell samples for the presence of the maternal and paternal alleles at various different loci on chromosome 11 and not on chromosome 11. In particular, Bischoff assayed for the presence of a maternal and paternal allele at the following loci:

1. two distinct loci located on the suspect "p" arm of chromosome pair 11, specifically the HBB and D11S904 loci;[48]

2. one locus on the opposite arm "q" of chromosome 11, specifically the CD3D locus; and

3. one locus on chromosome 21 (the INFAR locus)

As discussed below, Bischoff determined a first number and a second number of assay samples containing a selected and reference allele of interest, and ended up comparing these numbers with each other in order to ascertain an allelic imbalance.

(a) Bischoff's analysis involved "determin[ing] a first number of assay samples which contain a selected genetic sequence on a first chromosome and a second number of assay samples which contain a reference genetic sequence on a second chromosome"

Bischoff determined the presence of all maternal and paternal alleles at these loci and noted these down in Table 2, reproduced below.

[48] The analysis of both loci are redundant over each other, and Requester will focus mainly on the D11S904 locus going forward.

Table 2. Molecular analysis of single cells

Locus	Location	Mother	Father	Single cells						Interpretation[a]
				1	2	3	4	5	6	
HBB	11p15.5	2,3[b]	1,2	1,1	1,2	1,2	1,2	1,1	1,1	PID: 1,5,6; NBD: 2,3,4
D11S904	11p14–p13	2,4	1,3	1,1	1,4	1,4	1,4	1,1	1,1	PID: 1,5,6; NBD: 2,3,4
CD3D	11q23	2,3	1,3	1,2	1,2	1,2	1,2	1,2	1,2	NBD
INFAR	21q22.1	1,3	1,2	2,3	2,3	2,3	2,3	2,3	2,3	NBD

[a]PID = paternal isodisomy, NBD = normal biparental disomy, numbers correspond to individual single cells.
[b]Numbers represent alleles at each locus.

Table 2 indicates the identity of all alleles found in each of Bischoff's six single-cell samples. Table 2 presents the allelic status of all six single-cell samples at the four loci of interest. All four loci were "informative" for Bischoff's purposes as follows: for each locus, the patient carried two different alleles, one paternally-inherited and the other maternally-inherited.

At one locus (D11S904) the father and mother carried different non-inherited alleles, thus a total of four alleles at this locus were found in the parents (two different alleles that were inherited by the patient and two different non-inherited alleles), arbitrarily numbered alleles 1, 2, 3 and 4. In particular, the father carried D11S904 alleles 1 and 3 and the mother carried D11S904 alleles 2 and 4 and the patient inherited allele 1 from his father and allele 4 from his mother.

At the other three loci (HBB, CD3D and INFAR) the father and mother carried the same non-inherited allele, thus a total of three alleles at each locus were collectively found in both parents (two different alleles that were inherited by the patient and the same non-inherited allele), arbitrarily numbered alleles 1, 2 and 3. For the HBB locus, the father carried HBB alleles 1 and 2, the mother carried HBB alleles 2 and 3 and the patient inherited allele 1 from his father and allele 2 from his mother. For the CD3D locus, the father carried CD3D alleles 2 and 3, the mother carried CD3D alleles 1 and 3

and the patient inherited allele 2 from his father and allele 1 from his mother. For the INFAR locus, the father carried INFAR alleles 1 and 3, the mother carried INFAR alleles 1 and 2 and the patient inherited allele 3 from his father and allele 2 from his mother.

Bischoff first determined by karyotype analysis that all cells carried a maternal chromosome 11 and a paternal chromosome 11, both appearing to have an intact 11p arm ("High-resolution chromosome analysis revealed a normal 46, XY karyotype."[49]) But single-cell PCR simultaneously indicated that some cells were apparently missing maternal alleles within the 11p arm of maternal chromosome 11 (at loci D11S904 and HBB). Bischoff therefore inferred that in these cells, the 11p arm found to be present on the maternal chromosome 11 by karyotype analysis must have been derived from the paternal chromosome, and that the patient thus had two 11p regions both derived from the father.

Thus, under the broadest reasonable interpretation, Table 2 discloses a first number of assay samples containing a selected genetic sequence and a second number of assay samples containing a reference genetic sequence at each locus that Bischoff analyzed. Each of the single cells constitutes an assay sample as described previously, above. Each allele can be treated as a "*selected*" or a "*reference*" sequence in downstream comparisons. Treating for example the maternally-inherited D11S904 allele as the "selected genetic sequence" and the paternally-inherited D11S904 allele as the reference genetic sequence, there are 3 assay samples containing the selected genetic sequence and 6 assay samples containing the reference genetic sequence. Alternatively, treating the paternally-inherited D11S904 allele (designated D11S904 allele 1 in Table 2)

[49] Bischoff, page 398 ("Clinical history").

as the "selected genetic sequence" and the maternally-inherited D11S904 allele (designated D11S904 allele 4 in Table 2) as the reference genetic sequence, there are 6 assay samples containing the selected genetic sequence and 3 assay samples containing the reference genetic sequence.

Bischoff expressly determined the first and second numbers of assay samples at each locus. Regarding the 11p loci HBB and D11S904, Bischoff noted that "[t]hree of six cells showed paternal disomy [*i.e., two paternal and no maternal alleles*] with … two 11p markers" HBB and D11S904.[50] Bischoff further explained that in "cells numbered 1, 5 and 6 … only the paternal allele" was detected at either 11p locus HBB and D11S904 whereas "[n]ormal biparental inheritance [*of both maternal and paternal alleles*] was detected in cells 2, 3 and 4 with the [same] 11p markers."[51] Regarding the remaining "markers located outside of the BWS region, CD3D on 11q23 and INFAR on chromosome 21," Bischoff noted that there was "normal biparental inheritance in all single cells" at these markers, *i.e.*, all six assay samples contained the maternal allele and all six samples also contained the paternal allele of these markers.[52]

Regardless of which loci (HBB, D11S904, CD3D, and/or INFAR) are chosen, the results in Table 2 disclose the determination of a first number of assay samples containing a selected genetic sequence and a second number of assay samples containing a reference genetic sequence.

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[50] Bischoff, page 398, left col., top paragraph, and Table 2.
[51] Bischoff, page 397, left col., second paragraph , and Table 2.
[52] Bischoff, page 398, left col., top paragraph, and Table 2.

(b) Bischoff's analysis involved "comparing the first number ... to the second number of assay samples"

Bischoff also explicitly compared the first and second number of assay samples.

For the purposes of **Comparison 1 (between two non-homologous chromosomes)** identified in the overview section,[53] Bischoff determined that only three of the six cell samples ("*a first number of assay samples*") contained "*a selected genetic sequence on a first chromosome*" in the form of the maternally-inherited allele at the D11S904 locus (designated D11S904 allele 4 in Table 2) on the suspect "p" arm of maternal chromosome 11, while all six samples (a "*second number")* contained "*a reference genetic sequence on a second chromosome*" in the form of maternally-inherited allele INFAR (INFAR allele 3 in Table 2) on maternal chromosome 21 and compared these two numbers:

- Bischoff explained that "[t]hree of six cells showed paternal disomy [*i.e., presence of two paternal and absence of maternal alleles*] with … two 11p markers" HBB or D11S904, whereas "informative markers located outside of the BWS region … INFAR on chromosome 21, demonstrated normal biparental inheritance [*i.e., presence of both maternal and paternal alleles*] in all single cells with no intensity differences between alleles."[54]

- Bischoff again noted that in "cells numbered 1, 5 and 6 … only the paternal allele" was detected at either 11p locus (*i.e.*, HBB and D11S904) whereas "[n]ormal biparental inheritance [of both the maternal and paternal alleles] was detected in cells 2, 3 and 4 with the 11p markers and in all single cells … for the chromosome 21 marker, INFAR"[55]

- Bischoff elsewhere noted again that "paternal isodisomy of 11p [*i.e., presence of two paternal and absence of maternal alleles at 11p loci HBB or D11S904*] was detected in cells 1, 5 and 6 and normal biparental inheritance [*of a maternal and paternal allele*] of 11p in cells 2, 3 and 4."[56]

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[53] *See* Subsection (VI)(A)(1) above.
[54] Bischoff, page 398, left col., top paragraph, and Table 2.
[55] Bischoff, page 397, left col., second paragraph, and Table 2.
[56] Bischoff, page 397, left col., second paragraph, and Table 2.

In the absence of any allelic imbalance, one would have expected that each of the six samples would have shown a maternal allele at 11p loci such as HBB and D11S904, but Bischoff only found the maternal allele to be present in a subset of cells (cells 2-4).[57]

For the purposes of **Comparison 2 ("intra-locus," two homologous alleles on the suspect "p" arm of chromosome pair 11)** identified in the overview section, Bischoff determined that three of the six cell samples ("*a first number of assay samples*") contained "*a selected genetic sequence on a first chromosome*" in the form of the maternally-inherited allele at the D11S904 locus on the suspect "p" arm of maternal chromosome 11 (designated as D11S904 allele 4 in Table 2). In contrast, all six samples (a "*second number")* contained "*a reference genetic sequence on a second chromosome*" in the form of the paternally-inherited D11S904 allele (designated D11S904 allele 1 in Table 2), and compared these two numbers:

- Bischoff explained that "[t]hree of six cells showed paternal disomy [*i.e., presence of two paternal and absence of maternal alleles*] with … two 11p markers" HBB and D11S904 (also implicitly indicating that the remaining three cells showed both maternal and paternal alleles as normal)[58]

- Bischoff again noted that in "cells numbered 1, 5 and 6 … only the paternal allele" was detected at either 11p locus (*i.e.*, HBB and D11S904) whereas "[n]ormal biparental inheritance [*of both the maternal and paternal HBB or D11S904 alleles*] was detected in cells 2, 3 and 4 with the [same] 11p markers."[59]

- Bischoff elsewhere noted again that "paternal isodisomy of 11p [*i.e., presence of two paternal and absence of maternal alleles at 11p loci HBB or D11S904*] was detected in cells 1, 5 and 6 and normal biparental inheritance [*of a maternal and paternal allele*] of 11p in cells 2, 3 and 4."[60]

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[57] *See* Bischoff, page 397, Table 2).
[58] Bischoff, page 398, left col., top paragraph, and Table 2.
[59] Bischoff, page 397, left col., second paragraph , and Table 2.
[60] Bischoff, page 397, left col., second paragraph, and Table 2.

For the purposes of **Comparison 3 ("intra-locus," two homologous alleles on the non-suspect "q" arm of chromosome pair 11))** identified in the overview section, Bischoff determined that all six cell samples ("*a first number of assay samples*") contained "*a selected genetic sequence on a first chromosome*" in the form of the maternally-inherited allele at the CD3D locus on the non-suspect "q" arm of maternal chromosome 11 (designated CD3D allele 2 in Table 2), and also that all six samples (a "*second number of assay samples")* similarly contained "*a reference genetic sequence on a second chromosome*" in the form of the paternally-inherited CD3D allele (designated CD3D allele 1 in Table 2), and compared these two numbers:

- Bischoff explained that "informative markers located outside of the BWS region, CD3D on 11q23 … demonstrated normal biparental inheritance [*of both the maternal and paternal alleles*] in all single cells with no intensity differences between alleles."[61]

- Bischoff again noted that "[n]ormal biparental inheritance [*of both the maternal and paternal CD3D alleles*] was detected in … all single cells with the 11q marker, CD3D."[62]

For the purposes of **Comparison 4 ("intra-locus," two homologous alleles on non-suspect chromosome pair 21)** identified in the overview section, Bischoff determined that all six cell samples ("*a first number of assay samples*") contained "*a selected genetic sequence on a first chromosome*" in the form of the maternally-inherited allele at the INFAR locus on maternal chromosome 21 (designated INFAR allele 3 in Table 2), and also that all six samples (a "*second number*") similarly contained "*a reference genetic sequence on a second chromosome*" in the form of the paternally-

[61] Bischoff, page 398, left col., top paragraph, and Table 2.
[62] Bischoff, page 397, left col., second paragraph , and Table 2.

inherited INFAR allele on paternal chromosome 21 (designated INFAR allele 2 in Table 2), and compared these two numbers:

- Bischoff explained that "informative markers located outside of the BWS region, … [such as] INFAR on chromosome 21, demonstrated normal biparental inheritance [*of both the maternal and paternal INFAR alleles*] in all single cells with no intensity differences between alleles."[63]

- Bischoff again noted that "[n]ormal biparental inheritance [*of both the maternal and paternal INFAR alleles*] was detected in … all single cells … for the chromosome 21 marker, INFAR."[64]

Therefore, whether or not the "first chromosome" and "second chromosome" can be homologous to each other (as in comparisons 2-4, but not in comparison 1), Bischoff "***compare[ed]*** *the first number ... to the second number*", as recited in claim 1.

(c) Bischoff's comparison was done "to ascertain an allelic imbalance"

By comparing the numbers of assay samples containing a selected and reference sequence, Bischoff "*ascertain*[*ed*] *an allelic imbalance*" in the form of a loss of an allele in a subset of cells in a sample, as recited in claim 1.

Bischoff analyzed a patient with Beckwith-Wiedemann ("BWS") syndrome to determine the genetic event underlying the patient's condition.[65] Bischoff noted that some BWS patients had an allelic imbalance in the form of "partial paternal isodisomy of 11p"[66] Partial paternal 11p isodisomy (which Bischoff also referred to as 'uniparental disomy' or UPD) is a condition involving an aberrant unbalanced translocation of a

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[63] Bischoff, page 398, left col., top paragraph, and Table 2.
[64] Bischoff, page 397, left col., second paragraph , and Table 2.
[65] Bischoff, Abstract.
[66] Bischoff, Abstract.

chromosomal 11p region with subsequent retention of two copies of the paternal 11p region. In addition, the isodisomy in Bischoff's particular patient also "involve[ed] loss of the maternal 11p region in some cells,"[67] Thus both the paternal and maternal chromosomes of a chromosome pair each carry a copy of the paternal allele, and neither carries a copy of the maternal allele, of any locus located within the isodisomic portions of the chromosomes.

It should be noted that Bischoff 's paternal isodisomy involved two separate and distinct genetic aspects: first, loss of maternal 11p alleles and second, disomy (two copies of paternal 11p alleles). **Only the first genetic aspect of isodisomy (loss of maternal 11p alleles) is relevant to this request** – as explained below, Bischoff determined maternal allelic loss using the claimed methods.[68]

Because paternal 11p isodisomy involves loss of maternal alleles that were originally present on the "p" arm of chromosome 11, paternal isodisomy is an "allelic imbalance" under the broadest reasonable interpretation. Bischoff concluded the isodisomy was present in only a subset of cells because he observed "somatic mosaicism"[69] in which some cells in the patient sample were isodisomic and had lost the

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[67] Bischoff, page 398, left col., top paragraph. *See also* Fig. 3. depicting paternal isodisomy of the 11p arm ("PAT UPD 11p") as a recombinant chromosome pair 11, where both homologous chromosomes have a "p" arm derived from the father, and neither has a "p" arm derived from the mother.

[68] Although Bischoff also checked for disomy of paternal alleles, this analysis is not relevant to this request. By way of explanation, Bischoff determined disomy by karyotype analysis, which revealed that all cells appeared to carry two intact chromosomes 11, both appearing to have an intact 11p arm ("High-resolution chromosome analysis revealed a normal 46, XY karyotype.") Because single-cell PCR had indicated indicated that some cells were apparently missing maternal alleles within the 11p arm of maternal chromosome 11 (at loci D11S904 and HBB), Bischoff therefore inferred that in these cells, the 11p arm found to be present on the maternal chromosome 11 by karyotype analysis must have been derived from the paternal chromosome, and that the patient thus had two 11p regions both derived from the father.

[69] Bischoff, Abstract

maternal allele, but other cells in the sample were genetically normal with "normal biparental inheritance"[70] of both the maternal and paternal alleles. Table 2 illustrates this result: cells 1, 5 and 6 at 11p have two paternally-derived alleles and no maternal alleles whereas cells 2-4 have normal biparental distribution (NBD) at 11p and show one allele from each parent.[71] In particular, "[t]wo populations of cells were detected, a population of cells with normal biparental inheritance for chromosome 11 and a population of cells with partial paternal isodisomy of 11p."[72]

Because an allelic loss in a subset of cells in a sample is an "allelic imbalance" under the broadest reasonable interpretation, Bischoff's partial paternal isodisomy is an "allelic imbalance." Accordingly, Bischoff "*ascertain*[*ed*] *an allelic imbalance*" under the broadest reasonable interpretation.

v) In Bischoff's amplification methods, "between 0.1 and 0.9 of the assay samples yield[ed] an amplification product"

This cryptic recitation requires some claim interpretation. Claim 1 initially recites that "a population of amplified molecules" is generated in the amplifying step. Claim 1 also specifies in the analyzing step that a certain portion of samples yield "an amplification product" on analysis, instead of referring back to the "amplified molecules" recited in the amplifying step. The amplifying step does not contain any prior recitation of "an amplification product" that is recited in the analyzing step, such that the "*amplification product*" can be separate and distinct from the "*population of amplified molecules.*" In fact, if the claim is to be found valid, the "amplification product" of the

[70] Bischoff, Abstract
[71] Bischoff, page 397, Table 2.
[72] Bischoff, Abstract.

analyzing step must necessarily be separate and distinct from the "population of amplified molecules" of the amplifying step, at least because claim 1 requires that the "population of amplified molecules" is generated in "***each***" of the assay samples during the amplifying step, but also requires that between 0.1 and 0.9 (*i.e.*, ***not each***) of the assay samples yield "an amplification product" during the analyzing step.

As discussed above, Bischoff performed two separate and successive amplification reactions. The first amplification reaction was a whole-genome "PEP" amplification reaction that amplified the genomic template molecules in order "*to form a population of amplified molecules in each of the assay samples.*" The second amplification reaction was a locus-specific PCR reaction done as part of "analyzing" the PEP-amplified molecules. Thus, under the broadest reasonable meaning, Bischoff's secondary locus-specific amplification generates the "amplification product" mentioned in the analyzing step, which is separate and distinct from the "population of amplified molecules" generated by PEP whole-genome amplification in the preceding amplifying step.

Although claim 1 specifies that "0.1 to 0.9 of the assay samples yield an amplification product," claim 1 does not specify the particular template sequence from which the "amplification product" is derived. Bischoff's secondary locus-specific amplification reaction generated multiple different amplification products from different template sequences (loci). Requester notes that dependent claim 10, which is necessarily included within the scope of base claim 1, clarifies that between 0.1 and 0.9 of the assay samples yield an amplification product "as determined by amplification of the selected genetic sequence." Solely for the purposes of this reexamination, Requester will proceed

on the premise that by amplifying a "*selected genetic sequence*" in 0.5 (*i.e.*, between 0.1 and 0.9) of his assay samples as explained below, Bischoff necessarily anticipates dependent claim 10 and thereby also anticipates base claim 1, under the broadest reasonable interpretation.

Bischoff analyzed his PEP amplification products by a secondary analytical locus-specific PCR reaction, and found in "cells numbered 1, 5 and 6 … only the paternal allele" showed a (secondary) amplification product at the D11S904 locus whereas "[n]ormal biparental inheritance was detected" by generation of secondary amplification products of of both the maternal and paternal alleles "in cells 2, 3 and 4 with the [same] 11p markers."[73] Taking the maternal D11S904 allele as the "*selected genetic sequence on a first chromosome*," Bischoff found that only three ("*a first number*") of six single-cell assay samples apparently contained this allele. Thus, three of six (*i.e.*, 0.5) assay samples yielded an amplification product of the selected genetic sequence. Taking the maternal allele at the 11p locus HBB as the "*selected genetic sequence on a first chromosome*," yields the same result: three of six (*i.e.*, 0.5) assay samples were found to contain the selected genetic sequence. Because 0.5 is between 0.1 and 0.9, "*between 0.1 and 0.9 of the assay samples yield*[*ed*] *an amplification product*" from the secondary analytical amplification, as recited in claim 1.

Accordingly, Bischoff teaches that "*between 0.1 and 0.9 of the assay samples yield*[*ed*] *an amplification product*"" as recited in claim 1.

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[73] Bischoff, page 397, left col., second paragraph.

3. Detailed explanation of the pertinency and manner of applying Bischoff to independent claim 19

Independent claim 19 is substantially identical to independent claim 1, with only the following differences:

- Claim 19 recites a distributing/set-forming step before the amplifying step, *i.e.*, "distributing nucleic acid template molecules from a biological sample to form a set comprising a plurality of assay samples;"
- In contrast to claim 1, claim 19 does not require that amplification takes place in "each" assay sample;
- In contrast to claim 1, claim 19 does not require that "between 0.1 and 0.9 of the assay samples yield an amplification product" of a selected or reference sequence;
- Claim 19 explicitly specifies that the allelic imbalance is between the first chromosome (which bears the selected sequence) and the second chromosome (which bears the reference sequence)

i) Bischoff discloses "A method for determining an allelic imbalance in a biological sample, comprising the steps of:"

As explained with respect to claim 1 in Section (VI)(A)(2)(*i*), Bischoff discloses a "*method for determining an allelic imbalance in a biological sample*," as also recited in claim 19.

ii) *Bischoff discloses "distributing nucleic acid template molecules from a biological sample to form a set comprising a plurality of assay samples;"*

This distributing/set-forming step is not recited in claim 1. Bischoff, however, discloses such a step. Bischoff explains that to demonstrate allelic imbalance, a "blood sample was obtained for single cell analysis" from his patient, and "[b]y micromanipulation, single blood lymphocytes were isolated."[74] In particular, "[p]eripheral blood lymphocytes (uncultured) from the patient with BWS were individually visualized … and micromanipulated first into a wash droplet of DNA-free growth media and then placed into separate reaction tubes."[75]

Accordingly, Bischoff started with a biological sample (blood) and distributed single cells containing genomic template molecules into "separate reaction tubes," where each tube contained a single-cell assay sample. Accordingly, each tube contains nucleic acid template molecules from one peripheral blood lymphocyte. Bischoff thereby discloses "*distributing nucleic acid template molecules from a biological sample to form a set comprising a plurality of assay samples*" under the broadest reasonable interpretation.

iii) *Bischoff discloses "amplifying the template molecules within the assay samples to form a population of amplified molecules in the assay samples of the set; "*

As explained with respect to claim 1 in Section (VI)(A)(2)(*ii*), Bischoff discloses a "*amplifying the template molecules within the assay samples to form a population of amplified molecules in the assay samples of the set*," as also recited in claim 19.

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[74] Bischoff, sentence bridging pages 396-397.
[75] Bischoff, page 398, right col., section titled "Single cell micromanipulation and PEP."

iv) Bischoff discloses "analyzing the amplified molecules in the assay samples of the set"

As explained with respect to claim 1 in Section (VI)(A)(2)(*iii*), Bischoff discloses "*analyzing the amplified molecules in the assay samples of the set*," as also recited in claim 19.

*v) Bischoff's analysis involved both "**determin[ing]** a first number of assay samples which contain a selected genetic sequence on a first chromosome and a second number of assay samples which contain a reference genetic sequence on a second chromosome" and "**comparing** the first number ... to the second number of assay samples to ascertain an allelic imbalance ... in the biological sample."*

As explained with respect to claim 1 in Section (VI)(A)(2)(*iv*), Bischoff's analysis involved both "***determin[ing]*** *a first number of assay samples which contain a selected genetic sequence on a first chromosome and a second number of assay samples which contain a reference genetic sequence on a second chromosome" and "**comparing** the first number ... to the second number of assay samples to ascertain an allelic imbalance ... in the biological sample*," as also required by claim 19.

vi) Bischoff's purpose was to ascertain an allelic imbalance "between the first chromosome and the second chromosome"

In contrast to claim 1, claim 19 additionally specifies that the allelic imbalance is "*between the first chromosome and the second chromosome,*" which was not specified in claim 1. Under the broadest reasonable interpretation, this indicates that the first and second chromosomes can be homologous sister chromosomes of a chromosome pair, at least because sequences on non-homologous chromosomes are not normally alleles of

each other and thus an "allelic" imbalance does not normally exist "between" non-homologous chromosomes.

Bischoff ascertained an allelic imbalance "*between the first chromosome and the second chromosome* "as recited in claim 19. For example in **Comparison 2 ("intra-locus," two homologous alleles on the suspect "p" arm of chromosome pair 11)** identified in the overview section, Bischoff determined that three cell samples (*i.e.*, "*a first number*") contained the maternally-inherited allele at the D11S904 locus, situated on the suspect "p" arm of maternal chromosome 11 (*i.e.*, "*a first chromosome*"). In contrast, all six samples (a "*second number")* contained the paternally-inherited D11S904 allele on paternal chromosome 11 (*i.e.*, "*a second chromosome*"), and compared these two numbers.[76]

By ascertaining that an allelic imbalance existed between two sister alleles at the D11S904 locus, one situated on a "*first chromosome*" in the form of a maternal chromosome and the other allele situated on a "*second chromosome*" in the form of a homologous paternal chromosome, Bischoff thereby ascertained that an allelic imbalance existed between the first and second chromosomes.

Bischoff therefore ascertained an allelic imbalance "*between the first chromosome and the second chromosome,*" as recited in claim 19.

[76] *See*, *e.g.*, Bischoff, page 398, left col., top paragraph, and Table 2

4. Detailed explanation of the pertinency and manner of applying Bischoff to claims 5 and 22

Dependent claim 5 recites the method of claim 1 "*wherein the biological sample is from blood.*" Dependent claim 22 recites the method of claim 19 "*wherein the biological sample is from blood.*"

As explained above, Bischoff anticipates base claims 1 and 19. In addition, Bischoff started with a biological sample in the form of a "blood sample" and isolated single lymphocytes from this sample: "To determine whether somatic mosaicism was present in the patient, a … blood sample was obtained for single cell analysis. By micromanipulation, single blood lymphocytes were isolated …").[77]

Accordingly, Bischoff anticipates claims 5 and 22 as well as base claims 1 and 19.

5. Detailed explanation of the pertinency and manner of applying Bischoff to claims 8-15 and 20

Dependent claims 8-15 all recite the method of claim 1 wherein a specified subportion of the assay samples "*yield an amplification product*." Dependent claim 20 recites the method of claim 19 wherein a specified subportion of the assay samples "*yield an amplification product*." Under the broadest reasonable interpretation, Bischoff anticipates these claims.

i) *Anticipation of claims 8-15*

Dependent claims 8 and 9 specify that "*between 0.1 and 0.6*" (claim 8) or "*between 0.3 and 0.5*" (claim 9) "*of the assay samples yield an amplification product*," without specifying what particular template sequence the amplification product is generated from. Dependent claims 10, 12 and 14 specify that "*between 0.1 and 0.9*"

[77] Bischoff, page 396, right col., last paragraph (

(claim 10) or "*between 0.1 and 0.6*" (claim 12) or "*between 0.3 and 0.5*" (claim 14) "*of the assay samples yield an amplification product as determined by amplification of the selected genetic sequence*." Dependent claims 11, 13 and 15 specify that "*between 0.1 and 0.9*" (claim 11) or "*between 0.1 and 0.6*" (claim 13) or "*between 0.3 and 0.5*" (claim 15) "*of the assay samples yield an amplification product as determined by amplification of the reference genetic sequence*."

As discussed in the application of Bischoff to base claim 1 in Section (VI)(A)(2)(*v*), base claim 1 initially recites that "a population of amplified molecules" is generated in the amplifying step, but later specifies in the analyzing step that a certain portion of samples yield "an amplification product" instead of referring back to the "amplified molecules" recited in the amplifying step. In fact, "an amplification product" of the analyzing step must necessarily be separate and distinct from the "population of amplified molecules" of the amplifying step for this claim to be valid, at least because claim 1 requires that the "population of amplified molecules" is generated in "***each***" of the assay samples during the amplifying step, but also requires that between 0.1 and 0.9 (*i.e.*, ***not each***) of the assay samples yield "an amplification product" during the analyzing step.

As also discussed above, Bischoff performed two separate and successive amplification reactions. The first amplification reaction was a whole-genome "PEP" amplification reaction that amplified the genomic template molecules in order "*to form a population of amplified molecules in each of the assay samples.*" The second amplification reaction was a locus-specific PCR reaction, performed as part of "analyzing" the PEP-amplified molecules. Thus under the broadest reasonable meaning,

Bischoff's secondary locus-specific amplification is an analytical procedure that generates "an amplification product" as mentioned in the analyzing step, which is separate and distinct from the "population of amplified molecules" generated by PEP whole-genome amplification in the preceding amplifying step.

Bischoff analyzed his PEP amplification products by a secondary analytical locus-specific PCR reaction, and more specifically analyzed the maternal D11S904 allele (D11S904 allele 4 in Table 2) on chromosome 11, which he suspected was lost in a subset of cells. Bischoff found that only three ("*a first number*") of six single-cell assay samples yielded a (secondary) amplification product of the maternal HBB allele. In particular, Bischoff found in "cells numbered 1, 5 and 6 … only the paternal allele" showed a (secondary) amplification product at the D11S904 locus whereas "[n]ormal biparental inheritance" - *i.e.*, presence of both the maternal and paternal alleles - was detected "in cells 2, 3 and 4."[78] Thus, 0.5 (*i.e.*, three of six) assay samples yielded "an amplification product" of the maternal D11S904 allele.

Claims 8 and 9 specify that "*between 0.1 and 0.6*" (claim 8) or "*between 0.3 and 0.5*" (claim 9) "*of the assay samples yield an amplification product*" without specifying what particular template sequence the amplification product is generated from. Because these claims allow "*an amplification product*" to be amplified from any particular sequence including Bischoff's maternal D11S904 allele, and because Bischoff saw maternal D11S904 amplification product in 0.5 of his assay samples, Bischoff anticipates claims 8 and 9 in addition to base claim 1.

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[78] Bischoff, page 397, left col., second paragraph.

Dependent claims 10, 12 and 14 specify that "*between 0.1 and 0.9*" (claim 10) or "*between 0.1 and 0.6*" (claim 12) or "*between 0.3 and 0.5*" (claim 14) "*of the assay samples yield an amplification product as determined by amplification of the selected genetic sequence*." Bischoff made various comparisons between his maternal D11S904 allele and other sequences, thereby treating the maternal D11S904 allele as a "*selected genetic sequence*." Because Bischoff saw maternal D11S904 amplification product in 0.5 of his assay samples, where 0.5 is *"between 0.1 and 0.6"* (claim 8) or *"between 0.3 and 0.5"* (claim 9), Bischoff anticipates these claims in addition to base claim 1.

Dependent claims 11, 13 and 15 specify that "*between 0.1 and 0.9*" (claim 11) or "*between 0.1 and 0.6*" (claim 13) or "*between 0.3 and 0.5*" (claim 15) "*of the assay samples yield an amplification product as determined by amplification of the reference genetic sequence*." Bischoff made various comparisons between various sequences and his maternal D11S904 allele, thereby treating the maternal D11S904 allele (D11S904 allele 4 in Table 2) as a "*reference genetic sequence*" under the broadest reasonable interpretation. Because Bischoff saw maternal D11S904 amplification product in 0.5 of his assay samples, Bischoff anticipates these claims in addition to base claim 1. Accordingly, Bischoff anticipates dependent claims 8-15 in addition to base claim 1.

ii) *Anticipation of claim 20*

Claim 20 recites the method of independent claim 19, "*wherein between 0.1 and 0.9 of the assay samples yield an amplification product*."

Although base claim 19 recites that "*a population of amplified molecules*" is generated in the amplifying step, dependent claim 20 specifies that a certain portion of samples yield "an amplification product" instead of referring back to the "amplified

molecules" recited in the amplifying step of base claim 19. Thus base claim 19 does not contain any prior recitation of "*an amplification product*" that is recited in dependent claim 20, such that the "*amplification product*" recited in dependent claim 20 can be separate and distinct from the "*population of amplified molecules*" generated in the amplifying step of base claim 19

As also discussed above, "an amplification product" reads upon the secondary amplification products of Bischoff's secondary locus-specific PCR reactions performed after Bischoff's primary PEP amplification reaction. Analyzing the products of his secondary locus-specific amplification reaction, Bischoff found that three ("*a first number*") of six single-cell assay samples yielded a secondary amplification product of the maternal HBB allele. Thus, 0.5 (*i.e.*, three of six) assay samples yielded "an amplification product" of the maternal HBB allele.

Claim 20 specifies that "*between 0.1 and 0.9 of the assay samples yield an amplification product*" without specifying what particular template sequence the amplification product is generated from. Because claim 20 allows "*an amplification product*" to be amplified from any particular sequence including Bischoff's maternal HBB allele, and because Bischoff saw maternal HBB amplification product in 0.5 of his assay samples, Bischoff anticipates claim 20 in addition to base claim 19.

B. Proposed rejection 2: Bischoff renders obvious claims 2 and 3 in view of Kalinina under 35 U.S.C. § 103(a)

Dependent claim 2 recites the method of claim 1, wherein "*the step of amplifying employs real-time polymerase chain reactions*." Dependent claim 3 recites the method of

claim 2 "*wherein the real-time polymerase chain reactions comprise a dual-labeled fluorogenic probe*." Both claims are obvious over Bischoff in view of Kalinina.[79]

As discussed above, Bischoff anticipates base claim 1, by isolating single cells and performs a locus-specific amplification step. This amplification increases the amount of DNA sequence of interest for the subsequent analysis step, which uses hybridization with labeled sequence-specific probes.

Kalinina describes amplification of single-template molecules in nanoliter-volume samples, where amplification and analysis employ "*real-time polymerase chain reactions compris[ing] a dual-labeled fluorogenic probe*" as required by claims 2 and 3. The analysis method used in Kalinina is the well-known TaqMan® assay, in which dual-labelled TaqMan® probes are included within the amplification reaction mixture during the amplification procedure itself, and hybridize in real-time to a cognate amplification product as it is being generated. The probe used in Kalinina contains 6-carboxyfluorescein on the 5' end of the probe and 6-carboxytetramethylrhodamine on an internal nucleotide.[80] Both of these moieties are fluorogenic.[81] The '889 patent explicitly recognizes that TaqMan® probes are "dual-labeled fluorogenic probes,"[82] and are used in real-time PCR reactions.[83]

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[79] Kalinina *et al.*, *Nanoliter scale PCR with TaqMan detection*. Nucleic Acids Res. 25(10):1999-2004 (May 15, 1997), forming prior art to the '889 patent under 35 U.S.C. § 102(b) (Exhibit PA-2).

[80] Kalinina, page 2000 (Molecular biology reagents).

[81] *Id.* ("Typical values for average pixel intensity were ~ 130 relative fluorescence units (RFU) for fluorescein and ~ 60 RFU for rhodamine, with background emission from empty capillaries ~20 RFU at both wavelengths. In different experiments the fluorescein:rhodamine (F/R) ratio varied from ~1.0 to 2.0 in samples containing PCR product.")

[82] '889 patent, col. 7, lines 34-35.

[83] '889 patent, col. 5, lines 22-25.

In Kalinina's TaqMan® assay, PCR amplification of a sequence of interest is performed in the presence of an oligonucleotide probe labeled with a fluorescent reporter and a quencher molecule.[84] As amplification progresses, the dual-labeled probe will hybridize to the target sequence and the reporter molecule will be cleaved from the probe by Taq polymerase, resulting in an increase in fluorescence of the reporter.[85] The TaqMan® probe assay has the advantages of being more sensitive than conventional probe assays and better able to "detect PCR product derived from single template molecules,"[86] such as Bischoff's amplification products.

It would have been obvious to combine use of the TaqMan® assay described in Kalinina to detect the loci of interest described in Bischoff, under at least the following rationales:

Obviousness: Known Elements and Predictable Result

Under 35 U.S.C. § 103, where a claim "'simply rearranges old elements with each performing the same function it had been known to perform' and yields no more than what one would expect from such an arrangement, the combination is obvious." *KSR Intl. Co. v. Teleflex, Inc.*, 550 U.S. 398, 417 (2007), quoting *Sakraida v. Ag. Pro., Inc.*, 425 U.S. 273, 282 (1976).

Kalinina indicates that her methods are designed to "detect single starting template molecules," just as Bischoff's were as well (*e.g.*, Bischoff's methods were intended to detect the presence of a single template sequence molecule in the form of a

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[84] *Id.*
[85] *Id.*
[86] Kalinina at page 2003.

paternal 11p allele).[87] Kalinina's TaqMan® assay was specifically designed and optimized for use in single-molecule PCR assays such as Bischoff's. Both Bischoff and Kalinina both amplified and analyzed amplification products from a single template molecule.

Bischoff used two discrete amplification reactions: the first to amplify the locus of interest and the second to detect and analyze the amplified products. Performing two separate amplification reactions doubled the time, energy and resources needed for amplification, after which Bischoff's analysis procedure of overnight autoradiography required additional time. In contrast, Kalinina's use of the TaqMan® assay allowed both the amplification and the detection of the amplified products to occur in "*real time*" as recited in claim 2. Bischoff used a rather complicated procedure in order to amplify and analyze single templates in single cells (specifically, an amplifying step in the form of PEP amplification followed by any analyzing step including a locus-specific PCR reaction, and overnight autoradiography). In contrast, Kalinina's TaqMan® procedure was simpler and more efficient, combining both amplification and analysis in a single step in real time. Kalinina's amplification reaction would have been an obvious alternative to Bischoff's PEP amplification reaction as of the priority date of the '889 patent. Kalinina's data also indicated that Kalinina's TaqMan® amplification reaction was sensitive and efficient enough to always yield an amplification product from diploid-genome samples such as Bischoff's under the right conditions.[88] For example, Table 1 in

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[87] Kalinina, Abstract.

[88] Kalinina, Table 1. PCR reactions "were scored as positive if the maximum F/R ratio along the tube was ≥ 1.0" (page 2001, right col., second-last paragraph). Although Table 2 indicates that amplification was less efficient in larger capillaries, Kalinina teaches that the smaller the capillary size, the less efficient the amplification (*e.g.*, page 1999, right col., stating

Kalinina shows that for a small capillary diameter (25 or 50 μm), all assay samples containing the equivalent of 1.5 haploid genome equivalents yielded an amplification product (*i.e.*, the "[f]raction of capillaries with max F/R ≥ 1" was 1.0). The concentration of 1.5 haploid genomes per sample is slightly less than that of Bischoff, whose assay samples each contained 1 diploid genome (*i.e.*, the equivalent of 2 haploid genomes).

It would have been obvious to use Kalinina's amplification methods in a single-cell amplification procedure as taught by Bischoff. It should be noted that Bischoff's determined allelic imbalance by comparing numbers of each allele at a single locus of interest. Kalinina's TaqMan® assays were designed for exactly such analysis. Bischoff compared multiple different combinations of first and second allelic forms of a single marker, where each independent comparison was informative of allelic imbalance. At least some of these comparisons were limited to comparing alleles at a single locus (*e.g.*, the D11S904 locus at 11p) to check for a difference in numbers of assay samples indicative of an allelic imbalance at that locus.[89] Although Bischoff followed up by analyzing multiple different loci both inside and outside the imbalanced 11p portion of the genome, these comparisons were redundant over each other and merely served to locate the metes and bounds of the imbalanced genomic region (which is not a required step in the claims). Although analysis of multiple loci within a single cell is not required for the claimed methods, it would in any case have been obvious to perform Kalinina's

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that in order to "achieve single molecule sensitivity" with other PCR methods, generally "two or more sequential PCRs usually have to be performed, often using nested sets of primers … [w]e reasoned that the sensitivity of the TaqMan assay could be improved to enable detection of single starting molecules if reaction volumes were reduced").

[89] Bischoff, page 397, left col., ("In cells 1, 5 and 6, the 11p markers, HBB and D 11 S904, revealed the presence of only the paternal allele [but not the maternal allele at the same locus]… paternal isodisomy of 11p was detected in cells 1,5 and 6 and normal biparental inheritance of 11p in cells 2, 3 and 4.")

TaqMan® assays in a multiplexed format on Bischoff's single-cell samples if a skilled worker wished to analyze multiple loci within a single cell.

Thus, a person using Kalinina's amplification method on a single 11p locus would have arrived at the same conclusion as Bischoff, based on the same result (*i.e.*, that an allelic imbalance existed because the number of samples containing a maternal 11p allele were less than the number of samples containing an 11p paternal allele). The '889 claims therefore embody a merely predictable substitution of Kalinina's TaqMan® amplification procedure for Bischoff's PEP amplification procedure on Bischoff's single-cell samples.

Therefore, it would have been obvious to the skilled person to have used the TaqMan® assays to analyze the single cells and loci of Bischoff. Both Bischoff and Kalinina disclose the genetic analysis of very small quantities of starting genetic material, such as a single cell or single template. However, the TaqMan® assay, as discussed above, was a well-developed commercial assay with significant advantages over Bischoff, including the ability to perform both the amplification and the analysis in a single reaction container or receptacle. Furthermore, the '889 patent acknowledges that TaqMan® probes were commercially available by the priority date of the '706 patent, and that a skilled person would have been able to routinely implement the assay in Bischoff's system to obtain predictable results.[90] While Bischoff used a more complicated procedure in order to analyze multiple loci on a single cell (a PEP amplification followed by locus-specific PCR reaction, and overnight autoradiography) than Kalinina's TaqMan® procedure, it would have been obvious to amplify different single loci using TaqMan®

[90] '889 patent, col. 7, lines 32-37 ("Although the working examples demonstrate the use of molecular beacon probes as the means of analysis … other techniques can be used as well. These include … hybridization with other types of probes, including TaqManTM (dual-labeled fluorogenic) probes (Perkin Elmer Corp./Applied Biosystems, Foster City, Calif.),").

probes to arrive at the same results. Thus, claims 2 and 3 would have been *prima facie* obvious to one of ordinary skill in the art at the time of invention.

Obviousness: Reasons to Combine

Although a reason to use Kalinina's TaqMan® assay to generate Bischoff's single-genome assay samples is not required, an apparent reason to combine the known elements as claimed may be evidenced by the teachings of the references themselves, issues in the technical area, or the skill in the art. *KSR*, 550 U.S. at 418. Here, strong reasons to combine are directly provided by the references themselves.

Kalinina explicitly teaches the many advantages of TaqMan® assays, including that the "assay involves fluorescence measurements that can be performed without opening the PCR tube," and, as a result, "the risk of carry-over contamination is greatly reduced."[91] In particular, Kalinina details use of an improved PCR technique that would eliminate the need for the gel electrophoresis and subsequent autoradiography of Bischoff altogether by allowing for amplification and analysis in a single tube. While Bischoff used a more complicated procedure in order to analyze multiple loci on a single cell (a PEP amplification followed by locus-specific PCR reaction, and overnight autoradiography) than Kalinina's TaqMan® procedure, it would have been obvious to amplify different single loci in different cells using TaqMan® to arrive at the same results. It would have been *prima facie* obvious to one of ordinary skill in the art to modify the PCR method taught by Bischoff to use the TaqMan® single-tube PCR assays described in Kalinina to perform the amplification and detection/analysis of DNA sequences in cells with predictable results.

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[91] Kalinina at page 1999.

Obviousness: Known Technique to Improve Known Method

KSR and the MPEP provide that, where a known technique has been used to improve a base method ready for improvement, a POSITA would be capable of applying the known improvement to the base method.[92] Both Bischoff and Kalinina are directed to the use of PCR methods for molecular analysis of target nucleic acids. Kalinina recognizes that Taqman® assays impart single-molecule sensitivity to PCR reactions and reduce the risk of carry-over contamination."[93] Bischoff used a more complicated amplification-and-analysis workflow in order to analyze multiple loci on a single cell than Kalinina's TaqMan® procedure (specifically, a PEP amplification followed by locus-specific PCR reaction, and overnight autoradiography). But it would have been obvious to amplify different single loci in different cells using TaqMan® to arrive at the same results. Therefore, it would have been obvious to one of skill in the art to use the single tube assay techniques in Kalinina to improve the base assays of Bischoff with predictable results.

For at least these reasons, the combination of Bischoff and Kalinina renders claims 2-3 obvious.

C. Proposed rejection 3: Bischoff renders claims 4, 6 and 7 obvious under 35 U.S.C. § 103(a) in view of Zhang

Dependent claims 4, 6 and 7 recite the method of claim 1, "*wherein the selected genetic sequence"* or "*the reference genetic sequence*" or both sequences together is/are "*non-polymorphic*" marker(s). Although "non-polymorphic" is not defined in the '889

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92 *See* MPEP at §2143(C).
93 Kalinina at page 1999.

patent, during original prosecution the PTO took the view that a marker is "non-polymorphic" if different "allelic forms" of the marker do not exist.[94] Under this interpretation, claims 4, 6 and 7 are rendered obvious by the combination of Bischoff and Zhang.[95]

Bischoff anticipates base claim 1, whereas Zhang used single-cell PCR just like Bischoff using non-polymorphic markers as his "*selected*" and "*reference*" genetic sequences. Requester is concurrently applying Zhang as an anticipatory reference against substantially similar claims in a concurrent reexamination of a related patent No. 6,440,706 and the Examiner is referred to that concurrent request for a detailed explanation of how Zhang maps onto the substantially similar claims of the related '706 patent. Like Bischoff, Zhang isolated single cells from a biological sample, amplified different alleles at multiple loci in each cell,[96] and compared counts of different alleles, thereby amplifying, analyzing and comparing a first and second number of assay samples as required by claim 1. In addition, Zhang also amplified and compared "non-polymorphic" markers. Specifically, Zhang amplified and compared a "*selected genetic sequence*" in the form of "the STS gene on the X chromosome," and a "*reference genetic sequence*" in the form of "the STS pseudogene on the Y chromosome" to "determine the presence of the X or Y chromosome," and demonstrated that the "segregation pattern of … the X and Y chromosomes" showed "independent assortment of the sex

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[94] '889 prosecution history, Non-final Rejection mailed June 11, 2010, at page 4. (Exhibit 2).

[95] Zhang *et al., Whole genome amplification from a single cell: implications for genetic analysis*. PNAS USA, 89(13):5847-51 (1992) (Exhibit PA-3), forming prior art under 35 U.S.C. § 102(b) to the '889 patent.

[96] Zhang, page 5848, right col., second-last paragraph.

chromosomes."[97] Alternatively, the STS pseudogene on the Y chromosome could be treated as the "selected genetic sequence" and the STS gene on the X chromosome could be treated as the "reference genetic sequence" of the claims, arriving at the same result.

Zhang explains that the STS gene and the STS pseudogene are two different genes at two different loci on the X and Y chromosomes respectively.[98] Only one "allelic form" of each of these genes was present in Zhang's cells, which would be the case in cells derived from any male individual. Likewise, Bischoff's cells were derived from a male patient with one X and one Y chromosome,[99] such that both these genes (the X-linked STS gene and the Y-linked STS pseudogene) were "non-polymorphic" under the broadest reasonable interpretation.

It would have been obvious to assess genetic imbalance relating to the X and Y chromosomes by using Bischoff's single-cell PCR format with Zhang's suggested non-polymorphic markers (the X-linked STS gene and the Y-linked STS pseudogene), as explained below.

Obviousness: Reasons to Combine

Although a reason to combine Bischoff with Zhang is not required, an apparent reason to combine the known elements as claimed may be evidenced by the teachings of the references themselves, issues in the technical area, or the skill in the art. *KSR*, 550 U.S. at 418. Here, reasons to combine are directly provided by the references themselves. Bischoff demonstrated the feasibility of assessing genetic imbalance as an

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[97] Zhang, page 5848, right col., second-last paragraph.

[98] Zhang, page 5849, Legend for Fig. 3 ("The upper and lowere STS bands represent the Y chrosomome-linked (153 bp) and X chromosome-linked (144 bp) genes, respectively. . .).

[99] Bischoff, page 398, right col., "Clinical history" section ("High resolution chromosome analysis revealed a normal 46,XY karyotype").

underlying cause of diseases such as the Beckwith-Wiedemann syndrome by single-cell PCR. Various disorders such as Turner's syndrome are caused by an underlying genetic imbalance due to a loss of a sex chromosome. Zhang used two suitable non-polymorphic markers on the X and Y chromosomes for assessing sex-linked genetic imbalance. Thus, one of ordinary skill would have had ample reason to combine the teachings of Bischoff and Zhang in order to assess genetic imbalance relating to the X and Y chromosomes.

Accordingly, Bischoff renders claims 4, 6 and 7 obvious in view of Zhang.

D. Proposed rejection 4: Bischoff renders claims 16, 17 and 20 obvious in view of Li under 35 U.S.C. § 103(a)

Dependent claims 16 and 17 recite the method of claim 1, "*wherein the set comprises at least*" 500 (claim 16) or 1000 (claim 17) "*assay samples*." Dependent claim 20 recites the method of claim 19, "*wherein between 0.1 and 0.9 of the assay samples yield an amplification product*."

Under the broadest reasonable interpretation, Bischoff renders claims 16-17 and 20 obvious in view of Li.[100] Bischoff anticipates base claims 1 and 19 as explained previously. Li teaches or suggests single-cell PCR on greater than 500 or greater than 1000 assay samples, as recited in claims 16-17 respectively. Li also teaches single-cell PCR in which between 0.1 and 0.9 of the assay samples yield an amplification product, as recited in claim 20.

Specifically, Li teaches the method of single-cell PCR on both haploid (sperm) and diploid cells and made and analyzed a sets of single-cell assay samples by single-cell

[100] Li *et al.*, *Amplification and analysis of DNA sequences in single human sperm and diploid cells.* Nature. 29;335(6189):414-7 (1988), which forms prior art to the '889 patent under 35 U.S.C. § 102(b) (Exhibit PA-4).

PCR, and checked for imbalance in allelic representations in the form of segregation distortion in haploid cells.[101] Thus, both Bischoff and Li relate to the amplification and genotyping of single cells, in order to discover genetic imbalances in a biological sample, under the broadest reasonable interpretation.

i) Obviousness of claims 16-17

In prosecution of a pending continuation of the '889 patent, ***the PTO recently found that Li would have rendered it obvious to use a set of 500 or 1000 assay samples when amplifying and analyzing single-cell samples,***[102] just as Bischoff did. In particular, the PTO found that:

> Li expressly suggested analyzing 500 assay samples (page 416, last paragraph), and that it would have been *prima facie* obvious … to distribute 500, or even 1000 individual sperm [samples] 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 … Li's express contemplate[ion] [sic?] of 500 individual meiotic events certainly renders claim 63 obvious, and, by simple extrapolation, … [other claims] which merely require more assay samples (*i.e.*, 1000).

The PTO focused on Li's express teachings that determination of recombination frequency requires a large number of samples to get statistically significant results,

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[101] Li, page 415, right col., last paragraph. Li is being applied as an anticipatory reference against the claims of a parent patent No. 6,440,706 in a concurrent reexamination, and the Examiner is referred to the request filed in that reexamination for a detailed description of Li's teachings.

[102] Prosecution history of continuing App. No. 13/071,105 (Exhibit 11), Non-Final Office Action mailed October 10, 2012, at pages 7-8.

because recombination happens at a rate of 1% per million base pairs.[103] Li also teaches that his single-cell PCR methods are useful for a variety of purposes other than recombination frequency determination. For example, Li suggested that reliable analysis of "very large numbers" of single-cell samples would allow one to study "some mutational events which cannot be analysed by conventional methods."[104] A mild allelic imbalance showing infrequent loss of an allele is one exemplary mutational event to which single-cell PCR of "very large numbers" of cells could advantageously be applied. Accordingly, it would have been obvious to use Bischoff's allelic imbalance analysis on samples sets of over 500 and over 1000 samples as suggested by Li, especially in the case of subtle allelic imbalances, such as the somatic mosaicism (*i.e.*, allelic imbalance) of Bischoff. Thus, dependent claims 16 and 17 are *prima facie* obvious over Bischoff in view of Li.

ii) *Obviousness of claim 20*

Dependent claim 20 recites the method of claim 19, "*wherein between 0.1 and 0.9 of the assay samples yield an amplification product.*" Bischoff anticipates claim 19 using

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[103] Li, "Discussion" bridging pages 416-417 ("To date there have been no practical methods for accurate measurement of genetic distances of less than 1 cM. 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 between genetic markers which are physically very close. Because it should be possible to obtain statistically significant data on recombination frequencies from a single individual, it should also be possible to determine for the first time whether different individuals have the same or different rates of recombination for the same interval. … Pedigree analysis cannot measure recombination over the short intervals typical of many of the hot spot regions that have been deduced from population genetics data , given the number of informative families required and the effort involved in obtaining the data. With PCR, we can envisage typing as many as 500 meiotic products in a week. Decreasing the number of samples containing two sperm and increasing the efficiency of amplification of both loci simultaneously will be required for the highest resolution …").

[104] Li, page 417, left col., first paragraph.

a random whole-genome amplification method (PEP) to amplify sequences of interest as well as other irrelevant sequences in his single-cell assay sample, and analyzed the samples by means of a secondary locus-specific PCR reaction with gel electrophoresis and autoradiography. Li teaches an alternative and more straightforward method of single-cell amplification and analysis, which yields amplification products in "*between 0.1 and 0.9 of the assay samples*" as required by claim 20. In particular, Li amplified both alleles at a single locus (specifically, the globin gene) in single diploid-cell samples using a single set of primers and analyzed the products by hybridization with labeled "allele-specific oligonucleotide probes (ASO) which can distinguish between [the] two alleles."[105] Li found that this particular methodology showed a small sacrifice in efficiency over Bischoff's methods – in particular, "[o]ut of the 37 cells analysed" 84% of these samples hybridized with probes, indicating that Li's methodology was successful.[106] Because 84% (*i.e.*, 0.84) is "*between 0.1 and 0.9 of the assay samples,*" Li's amplification & analysis methodology would have yielded amplification products in "*between 0.1 and 0.9 of the assay samples*" as required by claim 20.

It would have been obvious to use Li's single-primer-pair amplification & analysis methods in Bischoff's single-cell analysis, under various rationales, as follows:

Obviousness: Known Elements and Predictable Result

Under 35 U.S.C. § 103, where a claim "'simply rearranges old elements with each performing the same function it had been known to perform' and yields no more than what one would expect from such an arrangement, the combination is obvious." *KSR*

[105] Li, page 414, right col., top paragraph ("Each individual cell was delivered into a PCR tube containing … PCR buffer … and ***a set of PCR primers*** that amplify the informative region of the globin gene … 50 cycles of amplification were performed").

[106] Li, page 414, right col., top paragraph.

Intl. Co. v. Teleflex, Inc., 550 U.S. 398, 417 (2007), *quoting Sakraida v. Ag. Pro., Inc.,* 425 U.S. 273, 282 (1976).

It would have been *prima facie* obvious to detect allelic loss at the D11S904 locus, as taught by Bischoff, using a single-primer-pair amplification format & analysis as taught by Li instead of Bischoff's PEP amplification and subsequent analysis. When considering obviousness of a combination of known elements, the operative question is "whether the improvement is more than the predictable use of prior art elements according to their established functions." *KSR*, 550 U.S. at 398; MPEP § 2141. Here, the methods of Bischoff and Li perform the same functions when operating together as each does separately, forming nothing more than a combination of well-known procedures in accordance with their intended functions.

Li's single-primer-pair PCR differed marginally from Bischoff's PEP amplification method in that Bischoff's "whole genome" method amplified multiple alleles from multiple loci from a single cell sample whereas Li's method amplified only a single locus of interest per sample. It would have been self-evident, however, to use Li's amplification methods on multiple single-cell samples to allow analysis of any number of loci. Perhaps more importantly, multiple-locus analysis is not relevant to the claimed methods or to Bischoff's determination of allelic imbalance *per se*. Bischoff compared multiple different combinations of selected and reference sequences, where each independent comparison was informative of allelic imbalance. At least some of these comparisons were between maternal and paternal alleles at a single locus to assess allelic imbalance. This is not surprising, since "allelic imbalance" can refer to an imbalance between maternal and paternal alleles within same locus. Not surprisingly, Bischoff

compared the maternal and paternal alleles at a *single* 11p locus (*e.g.*, the D11S904 locus at 11p) to determine whether a difference in counts existed between the two alleles, and based on the difference concluded that an allelic imbalance existed at that locus.[107] Although Bischoff also followed up this conclusion by analyzing multiple different loci both inside and outside the imbalanced 11p portion of the genome, these comparisons were redundant over each other and merely served to locate the metes and bounds of the imbalanced genomic region (which was found to be limited only to the "p" arm of chromosome 11 and did not extend to the "q" arm of chromosome 11 or to other chromosomes such as chromosome 21). Thus, a person using Li's amplification method on a single 11p locus would have arrived at the same results as Bischoff. The '889 claims therefore embody a merely predictable use of prior-art elements.

Obviousness: Reasons to Combine

Although a reason to combine Bischoff with Li is not required, an apparent reason to combine the known elements as claimed may be evidenced by the teachings of the references themselves, issues in the technical area, or the skill in the art. *KSR*, 550 U.S. at 418. Here, reasons to combine are evidenced by the references themselves. Both Bischoff and Li relate to the amplification and detection of alleles at a locus of interest. Bischoff used PEP whole-genome amplification followed by a locus-specific amplification with a single primer pair just as Li did. Li's method performed the single-locus amplification without first using a PEP whole-genome amplification step, which was unnecessary to determine allelic imbalance. When analyzing loci for allelic

[107] Bischoff, page 397, left col., ("In cells 1, 5 and 6, the 11p markers, HBB and D11S904, revealed the presence of only the paternal allele [but not the maternal allele at the same locus]… paternal isodisomy of 11p was detected in cells 1,5 and 6 and normal biparental inheritance of 11p in cells 2, 3 and 4.")

imbalance, it would thus have been obvious to one of ordinary skill to use Li's single-primer-pair PCR in Bischoff's single-cell analysis as being simpler, quicker and easier.

Thus, dependent claims 16, 17 and 20 are *prima facie* obvious over Bischoff in view of Li.

E. Proposed rejection 5: Bischoff renders claims 18, 20 and 21 obvious in view of Ruano II under 35 U.S.C. § 103(a)

Dependent claim 18 recites the method of claim 1 "*wherein the amplified molecules in each of the assay samples in the first and second numbers of assay samples are homogeneous such that the first number of assay samples do not contain the reference genetic sequence and the second number of assay samples do not contain the selected genetic sequence*." Dependent claim 20 recites the method of claim 19, "*wherein between 0.1 and 0.9 of the assay samples yield an amplification product*." Dependent claim 21 recites method of claim 20 "*wherein between 0.1 and 0.9 of the assay samples yield a homogeneous amplification product*." Bischoff anticipates base claims 1 and 19, as discussed above. In addition, Bischoff renders claims 18, 20 and 21 obvious in view of Ruano II[108] under the broadest reasonable interpretation.

In particular, Bischoff anticipates base claims 1 and 19 by teaching the use of single-cell PCR to distinguish between two polymorphic alleles at a given locus. Bischoff amplified both alleles in diploid cells simultaneously in a single reaction by PEP amplification and analyzed the PEP-amplified molecules by secondary locus-specific PCR in order to determine whether individual cells in his biological sample had lost an

[108] Ruano *et al.*, Nucleic Acids Res. 17(20):8392 (Oct 25, 1989), which forms prior art to the '889 patent under 35 U.S.C. § 102(b) (Exhibit PA-5).

11p maternal allele (*i.e.*, an allele on the "p" arm of maternal chromosome 11) and had thereby switched from a heterozygous allelic state to a hemizygous allelic state in which only the paternal allele was retained at this locus.

Ruano II teaches an amplification method which yields "*amplified molecules*" (claim 18) or an "*amplification product*" (claims 20 and 21) that meets the requirements of claims 18, 20 and 21. Ruano II teaches allele-specific PCR as an alternative amplification method that differentiates between two polymorphic alleles at a single locus.

Specifically, Ruano II used two different primer pairs in two separate PCR reactions, each pair capable of selectively amplifying only one allele and not the other. Ruano II studied a sample that was heterozygous at the globin gene locus, using a first primer pair having one primer "GR3" that annealed specifically to a first globin allele and the second primer pair having another primer "GR1" that annealed specifically to a second globin allele. For example, Ruano II states "the polymorphism defining the allele is at the 3' end of one of the two primers … The presence or absence of product after amplification with a given allele-specific primer … types the polymorphic priming site."[109] In Figure 1(b) shows the result of using primer pairs with GR1 or GR3 separately: two different amplification products were generated in separate samples.

Ruano II demonstrated that his allele-specific primers selectively amplified only its corresponding allele, so that every allele-specific PCR reaction generated a homogenous amplification product which did not contain any amplified molecules of the other allele despite being generated from a heterozygous sample with two different allelic

[109] Ruano II, page 8392, first paragraph.

templates (in Ruano's words, the amplification product was 'hemizygous' in content). In particular, Ruano II noted that "[w]hereas product amplified with 'GR=' [*i.e.*, non-allele-specific primer] is heterozygous, GR1 ASA [allele-specific amplification] product is hemizygous [*i.e.*, showing one allele rather than the usual two] for the upper band (-) and GR3 ASA product is hemizygous for the lower band (+)."[110] In contrast, the amplification product generated from non-allele-specific primer contained amplified copies of both alleles (in Ruano's words, the amplification product was "heterozygous").[111] The heterozygous amplification products amplified with the non-specific primer GR+ are shown in Figure 1(d). As a result, Ruano II could determine the allelotype of the globin locus "according to presence or absence of a … [PCR] product after ASA [*i.e.*, allele-specific amplification] with allele-specific primers GR1/GR3 and invariant primer GR5 (Fig 1b)."[112] For example, as stated by Ruano II, "Homozygote "A" sets the phase of one chromosome in "B" as 1,-; other chromosome is 3,+, which is inherited by "I" (homozygous 3,+) through "G". Therefore, the haplotypes are 1,- and 3,+."[113] Ruano's primers were designed to distinguish between two different polymorphic alleles that contained differing numbers of dinucleotide repeats (specifically, one allele had two "TG" repeats and the other allele had three such

[110] Ruano II, page 8392, third paragraph, and Fig. 1(b).
[111] Ruano II, page 8392, third paragraph, and Fig. 1(b).
[112] Ruano II, page 8392, second paragraph.
[113] Ruano II, page 8392, second paragraph.

repeats.[114] Bischoff similarly analyzed polymorphic loci that were also "dinucleotide repeat markers."[115]

Whereas Bischoff anticipates base claims 1 and 19, Ruano II's allele-specific amplification meets the added limitations of dependent claims 18, 20 and 21. Ruano II's allele-specific amplification yields "*amplified molecules*" (claim 18) or "*amplification product*" (claims 21) which is "*homogeneous*" as required by claims 18 and 21, and in particular is homogenous "*such that the first number of assay samples do not contain the reference genetic sequence and the second number of assay samples do not contain the selected genetic sequence*" as further specified by claim 21. In addition, when applied to Bischoff's single-cell samples, Ruano II's amplification methods would only generate an amplification product in "*between 0.1 and 0.9 of the assay samples*" as required by claim 20. In particular, amplification of a maternal allele in the 11p region would only produce an amplification product in half the assay samples, whereas amplification of a paternal allele at the same locus would product an amplification product in all the assay samples. Because Ruano II's methods would amplify the maternal and paternal alleles in separate reactions on separate samples, amplification of maternal and paternal alleles at an 11p locus would generate an amplification product in about 0.75 of the assay samples overall, *i.e.*, between 0.1 and 0.9 of the assay samples.

It would have been obvious to assess allelic imbalance using Bischoff's single-cell PCR strategy using allele specific primers as taught by Ruano II, where such primers

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[114] Ruano II, Fig. 1(b) legend (indicating that the GR1 primer sequence was GCTTTTCAC**(TG)**$_3$TCA and the GR3 primer sequence was AGCTTTTCAC**(TG)**$_2$TCAA).

[115] Bischoff, page 397, left col., top paragraph, describing the HBB, D11S904, CD3D and INFAR makers as four "informative dinucleotide repeat markers").

would amplify only a single allele, thereby producing a "*homogenous*" product as required by claims 18 and 21, under various rationales below.

Obviousness: Known Elements and Predictable Result

Under 35 U.S.C. § 103, where a claim "'simply rearranges old elements with each performing the same function it had been known to perform' and yields no more than what one would expect from such an arrangement, the combination is obvious." *KSR Intl. Co. v. Teleflex, Inc.*, 550 U.S. 398, 417 (2007), *quoting Sakraida v. Ag. Pro., Inc.,* 425 U.S. 273, 282 (1976).

It would have been *prima facie* obvious to detect allelic loss at the D11S904 locus, as taught by Bischoff, using an allele-specific amplification format as taught by Ruano II instead of Bischoff's PEP random whole-genome amplification procedure. When considering obviousness of a combination of known elements, the operative question is "whether the improvement is more than the predictable use of prior art elements according to their established functions." *KSR*, 550 U.S. at 398; MPEP § 2141. Here, the methods of Bischoff and Ruano II perform the same functions when operating together as each does separately, forming nothing more than a combination of well-known procedures in accordance with their intended functions.

Ruano II's allele-specific PCR differed marginally from Bischoff's PEP amplification method in that Bischoff's "whole genome" method amplified multiple alleles from multiple loci from a single cell sample whereas Ruano II's method amplified only one allele per sample. It would have been self-evident, however, to use Ruano II's amplification methods on multiple single-cell samples to allow analysis of any number of loci. Perhaps more importantly, multiple-locus analysis is not relevant to the claimed

methods or to Bischoff's determination of allelic imbalance *per se*. The term "allelic imbalance" at least sometimes refers to an imbalance between maternal and paternal alleles within a *single* locus under the broadest reasonable interpretation. Not surprisingly, Bischoff's determination of allelic imbalance was accordingly based on comparing the maternal and paternal alleles at a *single* 11p locus (either HBB or D11S904).[116] Although Bischoff also followed up this conclusion by analyzing multiple different loci both inside and outside the imbalanced 11p portion of the genome, these were redundant over each other and merely served to define the metes and bounds of the imbalanced genomic region (which was found to be limited only to the "p" arm of chromosome 11 and did not extend to the "q" arm of chromosome 11).

By using two separate allele-specific PCR reactions on two separate cells (one primer pair specific for the maternal allele and the other pair specific for the paternal allele) on a significant number of samples, a person using Ruano II's amplification method would have arrived at the same results as Bischoff. The '889 claims therefore embody a merely predictable use of prior-art elements.

Obviousness: Reasons to Combine

Although a reason to combine Bischoff with Ruano II is not required, an apparent reason to combine the known elements as claimed may be evidenced by the teachings of the references themselves, issues in the technical area, or the skill in the art. *KSR*, 550 U.S. at 418. Here, reasons to combine are evidenced by the references themselves. Both Bischoff and Ruano II relate to the amplification and detection of alleles at a locus of

[116] Bischoff, page 397, left col., ("In cells 1, 5 and 6, the 11p markers, HBB and D11S904, revealed the presence of only the paternal allele [but not the maternal allele at the same locus]… paternal isodisomy of 11p was detected in cells 1,5 and 6 and normal biparental inheritance of 11p in cells 2, 3 and 4.")

interest. Bischoff used PEP whole-genome amplification to amplify both alleles at a locus of interest within a single-cell assay sample, and could differentiate between each amplified allele due to a significant size difference,[117] and thus determined that three of his six cells had lost the maternal allele at an 11p locus. However, Bischoff's amplification method did not differentiate between amplified alleles of very similar size. In contrast, Ruano II's method differentiated between alleles of indistinguishable size (as shown Fig. 1(b) of Ruano), and can be practiced on other similarly-sized alleles. When analyzing loci with identically-sized alleles, it would have been obvious to one of ordinary skill to use Ruano II's allele-specific PCR in Bischoff's single-cell analysis to determine allelic imbalance.

Thus, dependent claims 18, 20 and 21 are *prima facie* obvious over Bischoff in view of Ruano II.

VII. CONCLUSION

Claims 1-22 of the '889 patent are anticipated under 35 U.S.C. § 102(b) over Bischoff, or alternatively rendered obvious over Bischoff. Accordingly, reexamination of claims 1-22 of the '889 patent is respectfully requested.

VIII. CONCURRENT LITIGATION AND REEXAMINATION PROCEEDINGS

The '889 patent is presently involved in litigation in the United States District Court for the Middle District of North Carolina Greensboro Division (Esoterix Genetic Laboratories, LLC and The Johns Hopkins University vs. Life Technologies Corporation,

[117] Bischoff, Figs. 1 and 2.

Applied Biosystems, LLC, and Ion Torrent Systems, Inc., Case No. 12-1173 (filed October 31, 2012)).

IX. AUTHORITY TO ACT AND CORRESPONDENCE ADDRESS

The real party in interest is Life Technologies Corporation, a Delaware corporation, having its principle place of business at 5791 Van Allen Way, Carlsbad, CA, 92008. Undersigned counsel states that it is acting on behalf of the real party in interest either in a representative capacity pursuant to C.F.R. §1.34(a), or under any power of attorney provided herewith.

Please send all correspondence to the address associated with customer number 52059, to the attention of: Legal – Intellectual Property Group, Life Tech Docket, Bldg. 5781, Office 8304.

X. REQUIRED FEES AND DEPOSIT ACCOUNT AUTHORIZATION

The Commissioner is authorized to charge the fee set forth in 37 C.F.R. §1.20(c)(1) to Life Technologies Deposit Account No. 50-3994. The Commissioner is authorized to charge any additional fees or credit any overpayment to Deposit Account No. 50-3994, as well as any and all other fees that have been or may be required from Requester, referencing Docket No. LT00831 REX 2.

Dated: June 17, 2013

Respectfully submitted,

By: /Ashita A. Doshi/

Reg. No. 57,327
Life Technologies Corporation
5791 Van Allen Way
Carlsbad, California 92008
(760) 845-2798

Electronic Patent Application Fee Transmittal

Application Number:	
Filing Date:	
Title of Invention:	Digital Amplification
First Named Inventor/Applicant Name:	Bert Vogelstein
Filer:	Ashita Amu Doshi/Elizabeth Morgan
Attorney Docket Number:	LT00831 REX 2

Filed as Large Entity

ex parte reexam Filing Fees

Description	Fee Code	Quantity	Amount	Sub-Total in USD($)
Basic Filing:				
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($)
Miscellaneous:				
			Total in USD ($)	**12000**

Electronic Acknowledgement Receipt

EFS ID:	16047189
Application Number:	90012895
International Application Number:	
Confirmation Number:	7285
Title of Invention:	Digital Amplification
First Named Inventor/Applicant Name:	Bert Vogelstein
Customer Number:	52059
Filer:	Ashita Amu Doshi/Elizabeth Morgan
Filer Authorized By:	Ashita Amu Doshi
Attorney Docket Number:	LT00831 REX 2
Receipt Date:	17-JUN-2013
Filing Date:	
Time Stamp:	16:40:29
Application Type:	Reexam (Third Party)

Payment information:

Submitted with Payment	yes
Payment Type	Deposit Account
Payment was successfully received in RAM	$12000
RAM confirmation Number	4052
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)/ Message Digest	Multi Part /.zip	Pages (if appl.)
1	Copy of patent for which reexamination is requested	LT00831REX2-Exhibit1-US7824889.pdf	22676464 b8b3fc472c5f0445bb3fb57cc8a8d7e92340bd8e	no	20
Warnings:					
Information:					
2	Reexam - Affidavit/Decl/Exhibit Filed by 3rd Party	LT00831REX2-Exhibit3-US7824889-file-history.pdf	8031421 96cc452ced28e1585ad3afec3482d831af15c73d	no	244
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Information:					
3	Reexam - Affidavit/Decl/Exhibit Filed by 3rd Party	LT00831REX2-Exhibit4-US6440706-file-history.pdf	9074494 06360611a451b51f1eba8fc0a1fc08766389e614	no	207
Warnings:					
Information:					
4	Reexam - Affidavit/Decl/Exhibit Filed by 3rd Party	LT00831REX2-Exhibit5-Lapidus-US5928870.pdf	1190615 404b32c76514ca6fbb2b16e058c9a002a6f472af	no	15
Warnings:					
Information:					
5	Reexam - Affidavit/Decl/Exhibit Filed by 3rd Party	LT00831REX2-Exhibit6-Ruano-1990.pdf	9472514 2ab7737b0542ffed5825dcdb1b069e79e3bfd80d	no	6
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Information:					
6	Reexam - Affidavit/Decl/Exhibit Filed by 3rd Party	LT00831REX2-Exhibit7-US7915015.pdf	1210854 900faa34263a26649e48bbc7e440f3b2abae695b	no	19
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Information:					
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Information:					
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12	Non Patent Literature	LT00831REX2-ExhibitPA-2-Kalinina-1997.pdf	9165021 c30a6e8ba71bcd8e16bd84addee2198495aa39d9	no	7
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16	Reexam - Info Disclosure Statement Filed by 3rd Party	LT00831REX2-Exhibit2-SB08.pdf	105728 58ad1d738801ca3d6c8997cc334d792160cc95e8	no	2
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17	Reexam Certificate of Service	LT00831REX2-certificate-service-reexam-request-6-17-13.pdf	87957 a3da6d4f2b7a3ea9a8ec4214ac1842641614c043	no	1
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Information:					
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Information:					
19	Receipt of Orig. Ex Parte Request by Third Party	LT00831REX2-reexam-request-6-17-13.pdf	418892 3b03aa97acd929754bf9befb649f836ab100c06e	no	78
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Information:					
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Information:					
		Total Files Size (in bytes):	104452095		

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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.

EXHIBIT 1

US007824889B2

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

(10) **Patent No.: US 7,824,889 B2**
(45) **Date of Patent: *Nov. 2, 2010**

(54) **DIGITAL AMPLIFICATION**

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

(73) Assignee: **The 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 659 days.

This patent is subject to a terminal disclaimer.

(21) Appl. No.: **11/709,742**

(22) Filed: **Feb. 23, 2007**

(65) **Prior Publication Data**

US 2008/0241830 A1 Oct. 2, 2008

Related U.S. Application Data

(60) Continuation of application No. 10/828,295, filed on Apr. 21, 2004, now abandoned, which is a division of application No. 09/981,356, filed on Oct. 12, 2001, now Pat. No. 6,753,147, which is a continuation of application No. 09/613,826, filed on Jul. 11, 2000, now Pat. No. 6,440,706.

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

(51) **Int. Cl.**
C12P 19/34 (2006.01)
C07H 21/04 (2006.01)

(52) **U.S. Cl.** **435/91.2**; 536/24.31; 536/24.33

(58) **Field of Classification Search** ..... None
See application file for complete search history.

(56) **References Cited**

U.S. PATENT DOCUMENTS

5,213,961 A 5/1993 Bunn et al.
5,518,901 A 5/1996 Murtagh
5,670,325 A 9/1997 Lapidus et al.
5,736,333 A 4/1998 Livak et al.
5,804,383 A 9/1998 Gruenert et al.
5,858,663 A 1/1999 Nisson et al.

(Continued)

FOREIGN PATENT DOCUMENTS

EP 0643140 A 3/1995

(Continued)

OTHER PUBLICATIONS

Loughlin et al. Association of the interleukin-1 gene cluster on chromosome 2q13 with knee osteoarthritis. Arthritis & Rheumatism 46(6):1519-1527, Jun. 2002.*

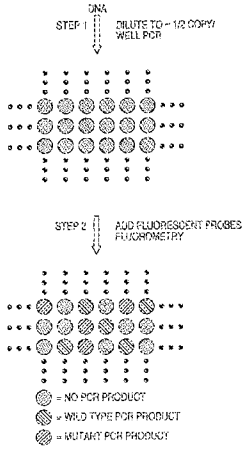
(Continued)

Primary Examiner—Samuel Woolwine
(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 pre-defined mutations. The process provides a reliable and quantitative measure of the proportion of variant sequences within a DNA sample.

22 Claims, 7 Drawing Sheets



U.S. PATENT DOCUMENTS

5,925,517	A	7/1999	Tyagi et al.
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.
6,143,496	A	11/2000	Brown et al.
6,291,163	B1	9/2001	Sidransky

FOREIGN PATENT DOCUMENTS

WO	WO 95/13399	5/1995
WO	WO 99/13113	3/1999

OTHER PUBLICATIONS

A. Piatek et al., "Molecular Beacon Sequence Analysis for Detecting Drug Resistance in Mycobacterium Tuberculosis", Nature Biotechnology, Apr. 1998, pp. 359-363, vol. 16, No. 4.

S. Tyagi et al., "Multicolor Molecular Beacons for allele discrimination", Nature Biotechnology, pp. 303-308, Jan. 1998, vol. 16, No. 1.

J. A.M. Vet et al., "Multilex Detection of Four Pathogenic Retroviruses Using Molecular Beacons", Proceedings of the National Academy of Sciences of the United States, May 25, 1999, 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, Jan. 15, 1999, pp. 181-191, vol. 266, No. 2.

B. Vogelstein et al., "Digital PCR", Proceedings of the National Academy of Sciences of the United States, Aug. 3, 1999, pp. 9236-9241, vol. 96, No. 16.

K. D.E. Everett et al, "Identification of nine species of the Chlamydiaceae Uisng PCR-RFLP", Apr. 1999, pp. 803-813, vol. 49, No. 2.

Darren G. Monckton, et al., "Minisatellite "Isoallele" Discrimination in Pseudohomozygotes by Single Molecule PCR and Variant Repeat Mapping", Genomics 11, pp. 465-467, 1991.

Gualberto Ruano, et al., "Haplotype of Multiple Polymorphisms Resolved by Enzymatic Amplification of Single DNA Molecules", Proc. National Science USA, 1990, vol 87 , pp. 6296-6300.

W. Navidi, et al., "Using PCR in Preimplantation Genetic Disease Diagnosis", Human Reproduction, vol. 6, No. 6, pp. 836-849, 1991.

Hongua Li, et al., "Amplification and Analysis of DNA Sequences in Single Human Sperm and Diploid Cells", Nature, vol. 335, Sep. 29, 1988, pp. 414-417.

Lin Zhang, et al., "Whole Genome Amplification from a Single Cell: Implications for Genetic Analysis", Proc. National Science USA, vol. 89, pp. 5847-5851, Jul. 1992.

David Sidransky, et al., "Clonal Expansion of p53 Mutant Cells is Associated with Brain Tumour Progression", Nature, Feb. 27, 1992, vol. 355, pp. 846-847.

Alec J. Jeffreys, et al., "Mutation Processes at Human Minisatellites", Electophoresis, 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", Forensic Science International, vol. 66, pp. 129-141, 1994.

Paul M. Lizardi, et al., "Mutation Detection and Single-Molecule Counting Using Isothermal Rolling-Circle Amplification", Nature Genetics, vol. 19, Jul. 1998, pp. 225-232.

R. Parsons, et al., "Mismatch Repair Deficiency in Phenotypically Normal Human Cells", Science, vol. 268, May 5 1995, pp. 738-740.

Marras et al., "Multiplex Detection of Single-Nucleotide Variations Using Molecular Beacons," Genetic Analysis: Biomolecular Engineering, Feb. 1999, 14; 151-156.

Whitcomb et al., "Detection of PCR Products Using Self-Probing Amplicons and Fluorescence," Nature Biotechnology, Aug. 1999, vol. 17, 804-807.

P. J. Sykes, "Quantitation of Targets for PCR by Use of Limiting Dilution," BioTechniques, (1992), vol. 13, No. 3, pp. 444-449.

M.J. Brisco et al., "Detection and Quantitation of Neoplastic Cells in Acute Lymphoblastic Leukaemia, by Use of the Polymerase Chain Reaction," British Journal of Haematology, 1991, 79, 211-217.

M. J. Brisco et al., "Outcome Prediction in Childhood Acute Lymphoblastic Leukaemia by Molecular Quantification of Residual Disease at the End of Induction," The Lancet, Jan. 22, 1994, vol. 343, pp. 196-200.

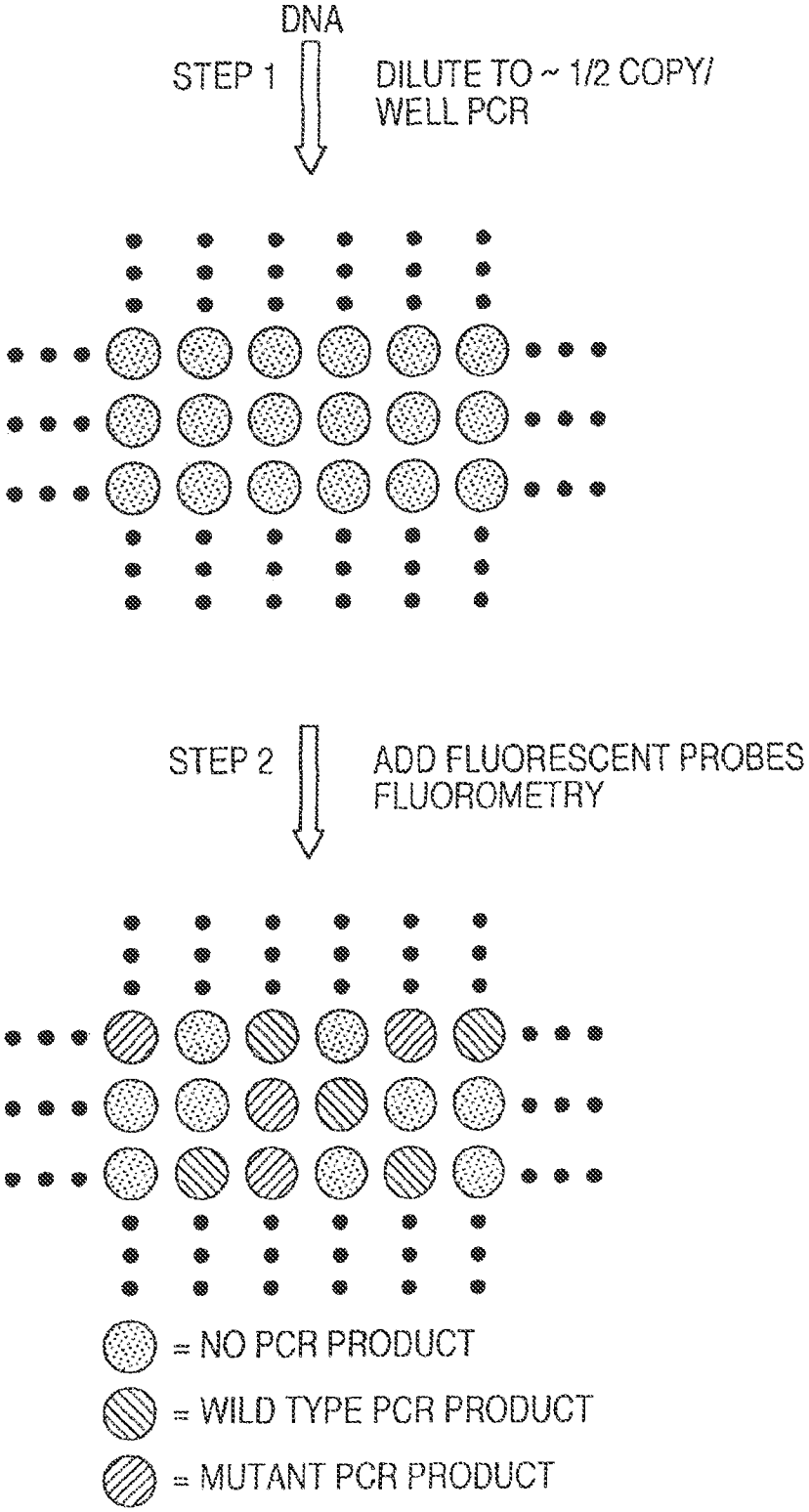
Newton, PCR Essential Data, pp. 51-52, 1995.

Notice of Reasons for Rejection dispatched Apr. 28, 2010 in Japanese Application No. 2001-513641 and English translation thereof.

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).

* cited by examiner

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

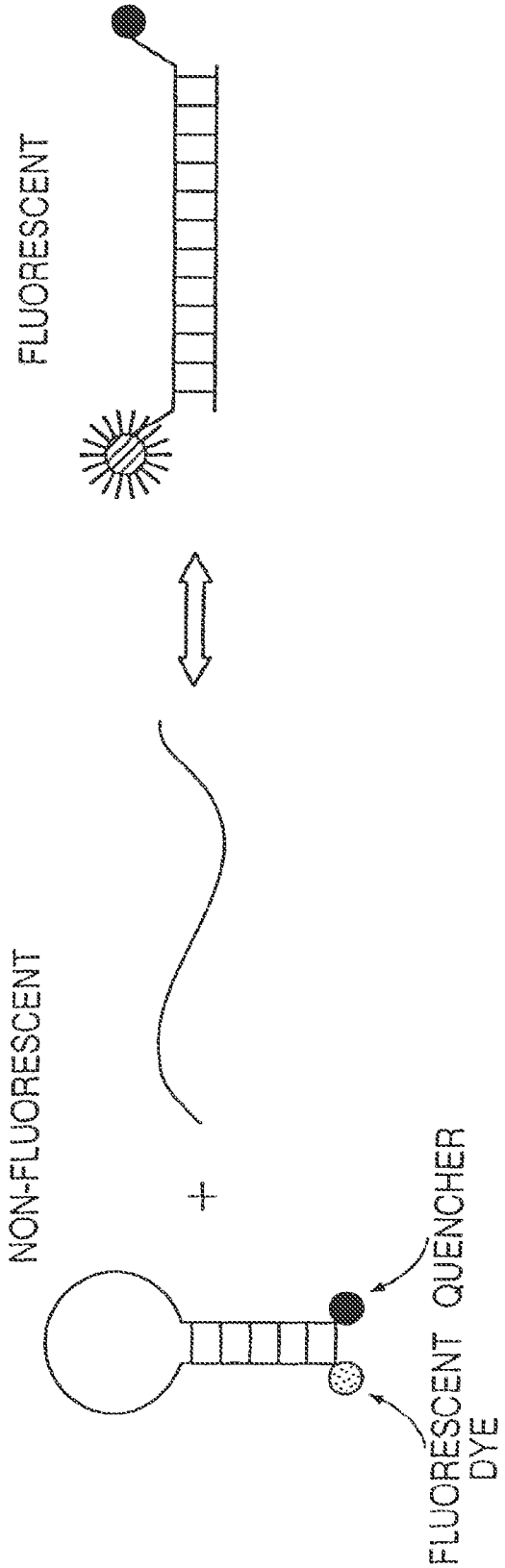


FIG. 1B

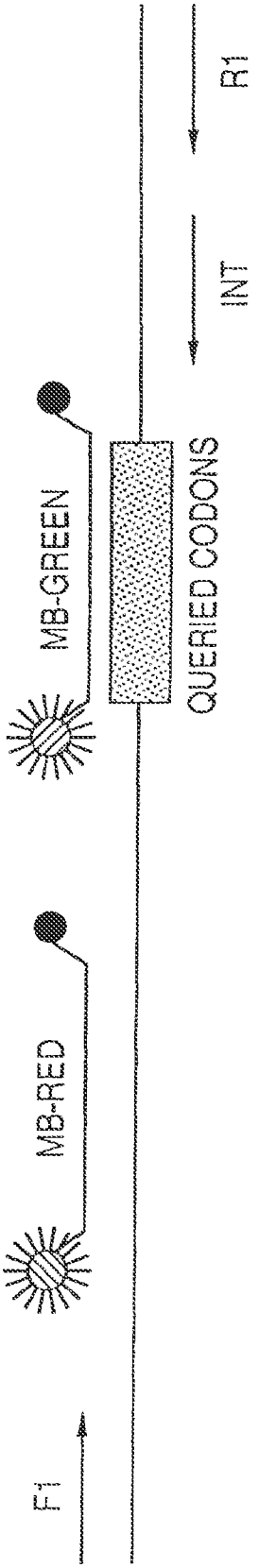
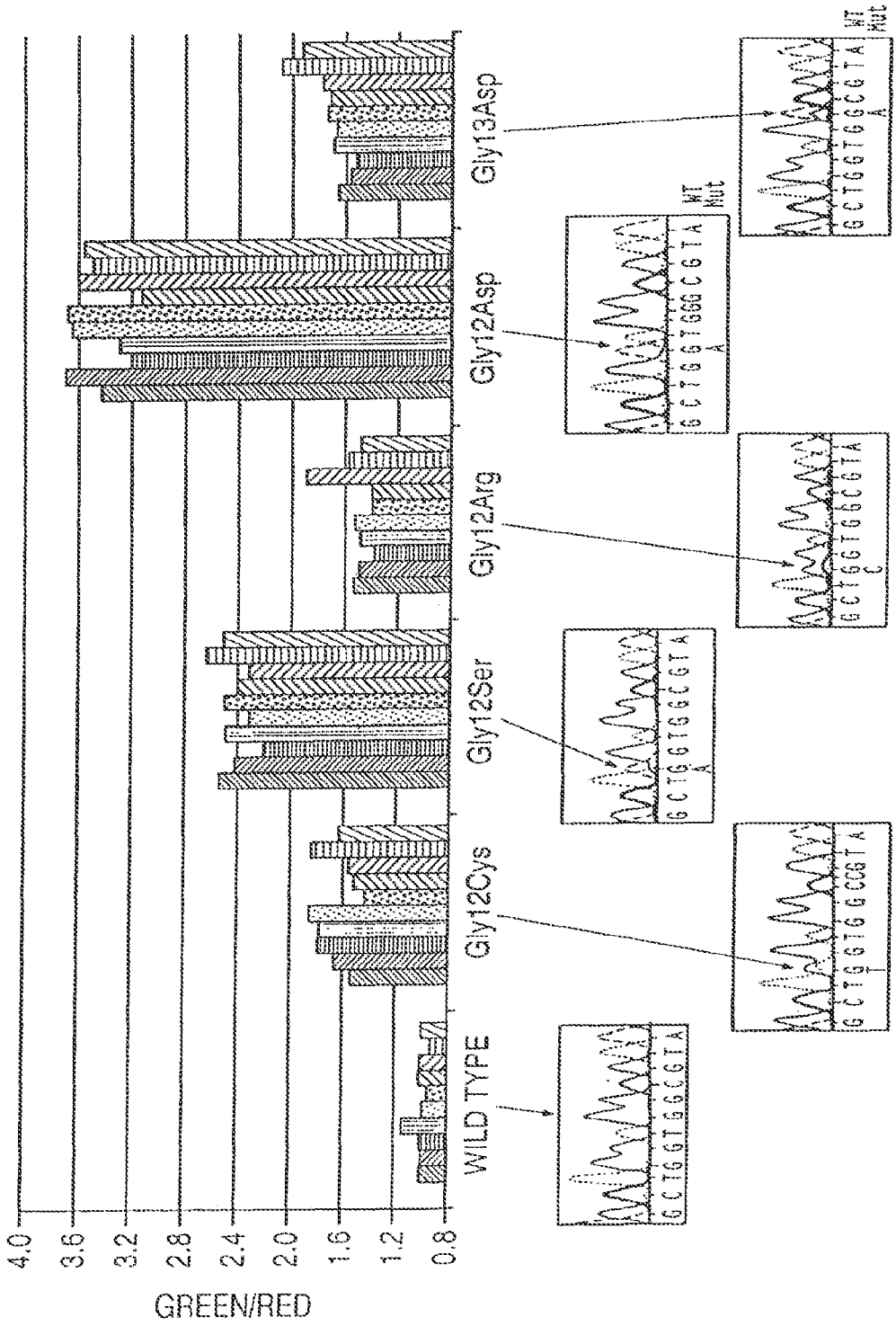


FIG. 1C

FIG. 2


4.0
3.6
3.2
2.8
2.4
2.0
1.6
1.2
0.8
GREEN/RED
WILD TYPE
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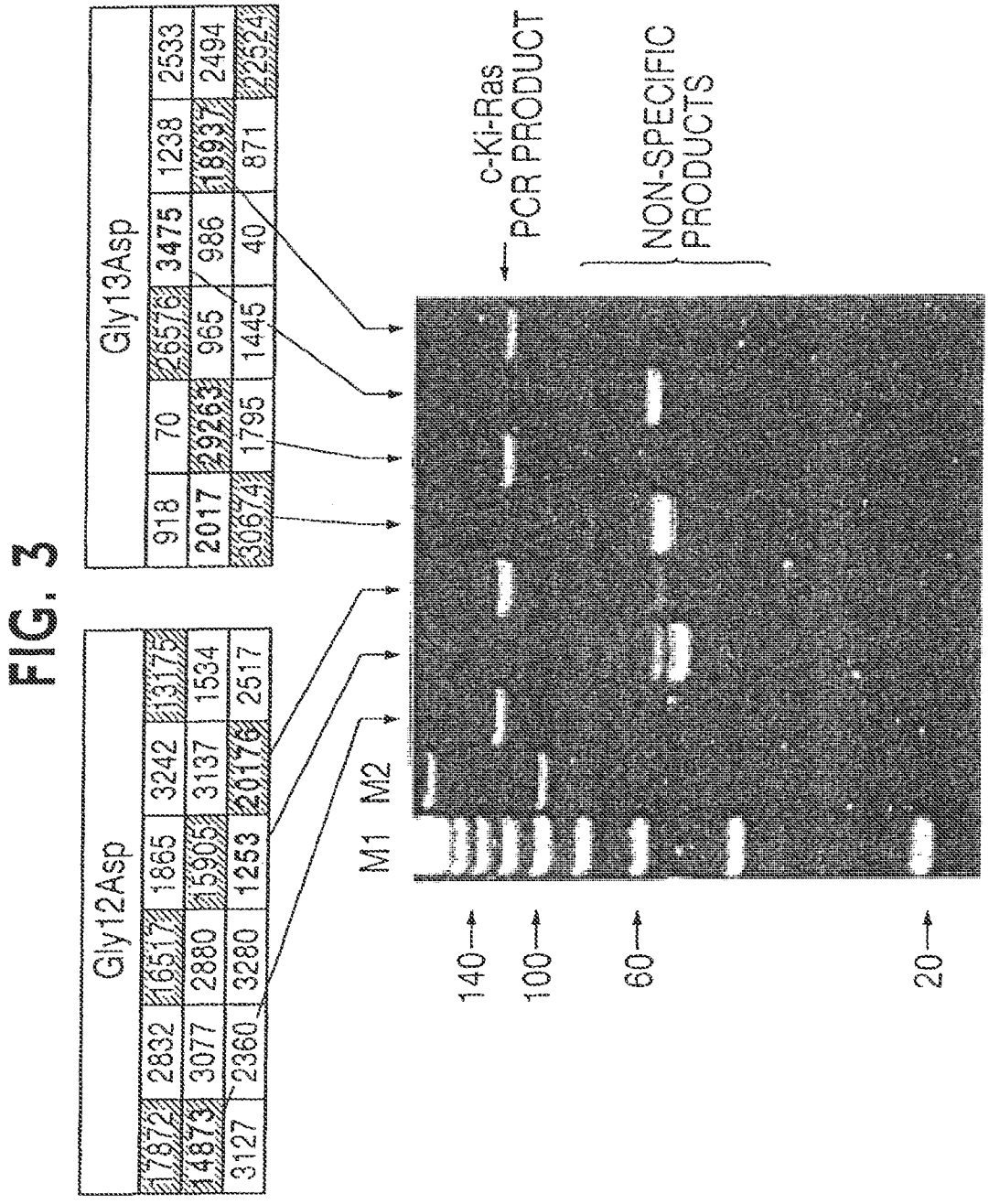
Gly12Ser
Gly12Arg
Gly12Asp
Gly13Asp
WT
Mut

FIG. 3


Gly12Asp
78722
2832
165174
1865
3242
13175
48736
3077
2880
59052
3137
1534
3127
2360
3280
12530
20176
2517
Gly13Asp
918
70
26576
3475
1238
2533
2017
29263
965
986
18937
2494
30674
1795
1445
40
871
22524
M1
M2
c-Ki-Ras PCR PRODUCT
NON-SPECIFIC PRODUCTS
140
100
60
20

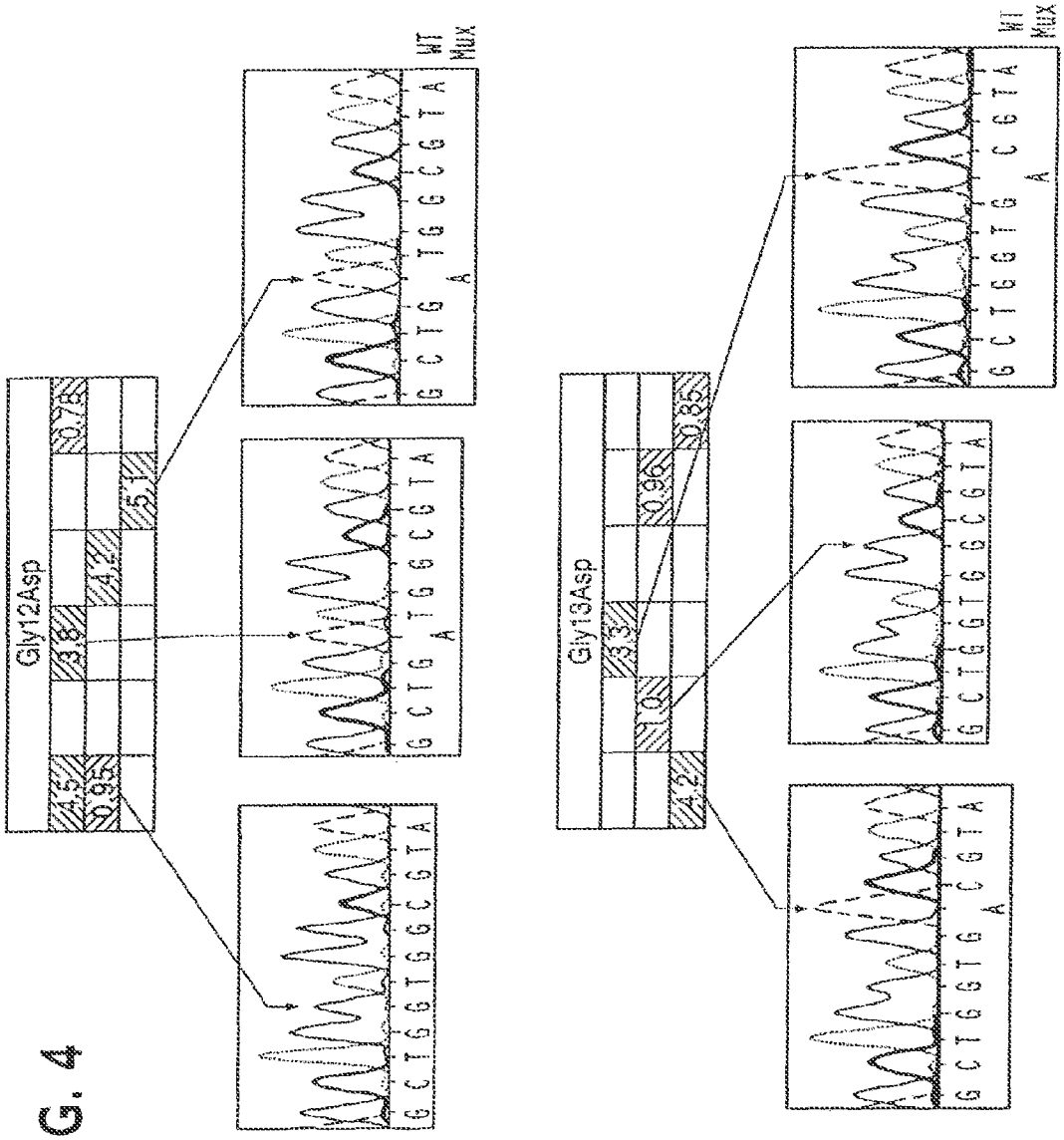
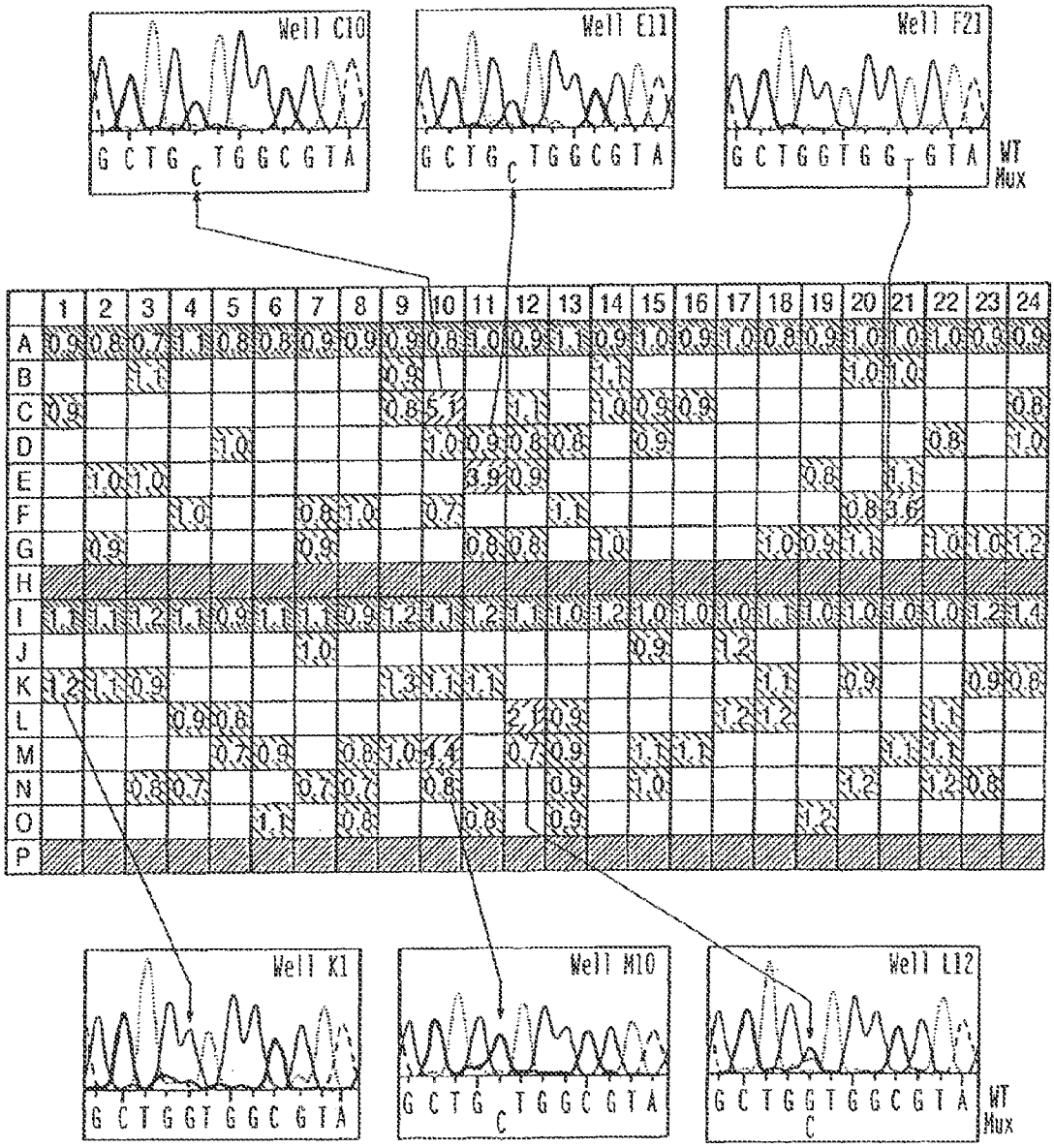

Gly12Asp
2.5
0.95
3.8
4.2
5.1
0.78
G C T G T G G C G T A
G C T G A T G G C G T A
G C T G A T G G C G T A
WT
Mux
Gly13Asp
3.3
1.0
2.2
0.96
0.85
G C T G G T G C G T A
G C T G G T G C G T A
G C T G G T G A G T A
WT
Mux

FIG. 4

FIG. 5


Well C10
G C T G C T G G C G T A
Well E11
G C T G C T G G C G T A
Well F21
G C T G G T G G T G T A
WT Mux
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20 21 22 23 24
A
B
C
D
E
F
G
H
I
J
K
L
M
N
O
P
Well K1
G C T G G T G G C G T A
Well M10
G C T G C T G G C G T A
Well L12
G C T G G T G G C G T A
WT Mux

DIGITAL AMPLIFICATION

This application is a continuation of U.S. application Ser. No. 10/828,295 filed Apr. 21, 2004, which is a divisional of U.S. application Ser. No. 09/981,356 filed Oct. 12, 2001, now U.S. Pat. No. 6,753,147, which is a continuation of U.S. application Ser. No. 09/613,826 filed Jul. 11, 2000, now U.S. Pat. No. 6,440,706, which claims the benefit of provisional U.S. Application Ser. No. 60/146,792, filed Aug. 2, 1999. The disclosure of all priority applications is expressly incorporated herein.

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.

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

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

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

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

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

BRIEF DESCRIPTION OF THE DRAWINGS

FIGS. 1A, 1B, 1C. Schematic of experimental design. (FIG. 1A) The basic two steps involved: PCR on diluted DNA samples is followed by addition of fluorescent probes which discriminate between WT and mutant alleles and subsequent fluorometry. (FIG. 1B) Principle of molecular beacon analy-

sis. In the stem-loop configuration, fluorescence from a dye at the 5' end of the oligonucleotide probe is quenched by a Dabcyl group at the 3' end. Upon hybridization to a template, the dye is separated from the quencher, resulting in increased fluorescence. Modified from Marras et al. (FIG. 1C) Oligonucleotide design. Primers F1 and R1 are used to amplify the genomic region of interest. Primer INT is used to produce single stranded DNA from the original PCR products during a subsequent asymmetric PCR step (see Materials and Methods). MB-RED is a Molecular Beacon which detects any appropriate PCR product, whether it is WT or mutant at the queried codons. MB-GREEN is a Molecular Beacon which preferentially detects the WT PCR product.

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

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

FIG. **4**. Discriminating WT from mutant PCR products obtained in Dig-PCR. RED/GREEN ratios were determined from the fluorescence of MB-RED and MB-GREEN as described in Materials and Methods. The wells shown are the same as those illustrated in FIG. **3**. The sequences of PCR products from the indicated wells were determined as described in Materials and Methods. The wells with RED/GREEN ratios >3.0 each contained mutant sequences while those with RED/GREEN ratios of ~1.0 contained WT sequences. WT c-Ki-Ras (SEQ ID NO: 7), Gly12Asp (SEQ ID NO: 13), and Gly13Asp (SEQ ID NO: 9) were analyzed.

FIG. **5**. Dig-PCR of DNA from a stool sample. The 384 wells used in the experiment are displayed. Those colored blue contained 25 genome equivalents of DNA from normal cells. Each of these registered positive with MB-RED and the RED/GREEN ratios were 1.0+/−0.1 (mean +/−1 standard deviation). The wells colored yellow contained no template DNA and each was negative with MB-RED (i.e., fluorescence <3500 fluorescence units.). The other wells contained diluted DNA from the stool sample. Those registering as positive with MB-RED were colored either red or green, depending on their RED/GREEN ratios. Those registering negative with MB-RED were colored white. PCR products from the indicated wells were used for automated sequence analysis. The sequence of WT c-Ki-Ras in well 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 so that the resultant products have a proportion of the analyte sequence which is detectable by the detection means chosen. At its limit, single template molecules can be amplified so that the products are completely mutant or completely wild-type (WT). The homogeneity of these amplification products makes them trivial to distinguish through existing techniques.

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

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

Digital amplification can be used to detect mutations present at relatively low levels in the samples to be analyzed. The limit of detection is defined by the number of wells that can be analyzed and the intrinsic mutation rate of the polymerase used for amplification. 384 well PCR plates are commercially available and 1536 well plates are on the horizon, theoretically allowing sensitivities for mutation detection at the ~0.1% level. It is also possible that Digital Amplification can be performed in microarray format, potentially increasing the sensitivity by another order of magnitude. This sensitivity may ultimately be limited by polymerase errors. The effective error rate in PCR as performed under our conditions was 1.1%, i.e., four out of 351 PCR products derived from WT DNA sequence appeared to contain a mutation by RED/GREEN ratio criteria. However, any individual mutation (such as a G to T transversion at the second position of codon 12 of c-Ki-Ras), are expected to occur in <1 in 50 of these polymerase-generated mutants (there are at least 50 base substitutions within or surrounding codons 12 and 13 that should yield high RED/GREEN ratios). Determining the sequence of the putative mutants in the positive wells, by direct sequencing as performed here or by any of the other techniques, provides unequivocal validation of a prospective mutation: a significant fraction of the mutations found in individual wells should be identical if the mutation occurred in vivo. Significance can be established through rigorous

statistical analysis, as positive signals should be distributed according to Poisson probabilities. Moreover, the error rate in particular Digital Amplification experiments can be precisely determined through performance of Digital Amplification on DNA templates from normal cells.

Digital Amplification is as easily applied to RT-PCR products generated from RNA templates as it is to genomic DNA. For example, the fraction of alternatively spliced or mutant transcripts from a gene can be easily determined using photoluminescent probes specific for each of the PCR products generated. Similarly, Digital Amplification can be used to quantitate relative levels of gene expression within an RNA population. For this amplification, each well would contain primers which are used to amplify a reference transcript expressed constitutively as well as primers specific for the experimental transcript. One photoluminescent probe would then be used to detect PCR products from the reference transcript and a second photoluminescent probe used for the test transcript. The number of wells in which the test transcript is amplified divided by the number of wells in which the reference transcript is amplified provides a quantitative measure of gene expression. Another group of examples involves the investigations of allelic status when two mutations are observed upon sequence analysis of a standard DNA sample. To distinguish whether one variant is present in each allele (vs. both occurring in one allele), cloning of PCR products is generally performed. The approach described here would simplify the analysis by eliminating the need for cloning. Other potential applications of Digital Amplification are listed in Table 1. When the goal is the quantitation of the proportion of two relatively common alleles or transcripts rather than the detection of rare alleles, techniques such as those employing TaqMan and real time PCR provide an excellent alternative to use of molecular beacons. Advantages of real time PCR methods include their simplicity and the ability to analyze multiple samples simultaneously. However, Digital Amplification may prove useful for these applications when the expected differences are small, (e.g., only ~2-fold, such as occurs with allelic imbalances.)

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 mutant alleles both vs. mutations in the same allele	first mutation	second mutation
Allelic Imbalance	Quantitative analysis with non-polymorphic markers	marker sequence	marker from another chromosome

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

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

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

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

The number of different situations in which the digital amplification method will find application is large. Some of these are listed in Table 1. As shown in the examples, the method can be used to find a tumor mutation in a population of cells which is not purely tumor cells. As described in the examples, a probe for a particular mutation need not be used, but diminution in binding to a wild-type probe can be used as an indicator of the presence of one or more mutations. Chromosomal translocations which are characteristic of leukemias or lymphomas can be detected as a measure of the efficacy of therapy. Gene amplifications are characteristic of certain disease states. These can be measured using digital amplification. Alternatively spliced forms of a transcript can be detected and quantitated relative to other forms of the transcript using digital amplification on cDNA made from mRNA. Similarly, using cDNA made from mRNA one can determine relative levels of transcription of two different genes. One can use digital amplification to distinguish between a situation where one allele carries two mutations and one mutation is carried on each of two alleles in an individual. Allelic imbalances often result from a disease state. These can be detected using digital amplification.

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

Molecular beacon probes according to the present invention can utilize any photoluminescent moiety as a detectable moiety. Typically these are dyes. Often these are fluorescent dyes. Photoluminescence is any process in which a material is excited by radiation such as light, is raised to an excited

electronic or vibronic state, and subsequently re-emits that excitation energy as a photon of light. Such processes include fluorescence, which denotes emission accompanying descent from an excited state with paired electrons (a "singlet" state) or unpaired electrons (a "triplet" state) to a lower state with the same multiplicity, i.e., a quantum-mechanically "allowed" transition. Photoluminescence also includes phosphorescence which denotes emission accompanying descent from an excited triplet or singlet state to a lower state of different multiplicity, i.e., a quantum mechanically "forbidden" transition. Compared to "allowed" transitions, "forbidden" transitions are associated with relatively longer excited state lifetimes.

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

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

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

EXAMPLE 1

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

EXAMPLE 2

Step 2: Fluorescence analysis. 3.5 ul of a solution with the following composition was added to each well: 67 mM Tris, pH 8.8, 16.6 mM NH_4SO_4, 6.7 mM $MgCl_2$, 10 mM β-mercaptoethanol, 1 mM dATP, 1 mM dCTP, 1 mM dGTP, 1 mM TTP, 6% DMSO, 5 uM primer INT, 1 uM MB-GREEN, 1 uM MB-RED, 0.1 units/ul Platinum Taq polymerase. The plates were centrifuged for 20 seconds at 6000 g and fluorescence read at excitation/emission wavelengths of 485 nm/530 nm for MB-GREEN and 530 nm/590 nm for MB-RED. This fluorescence in wells without template was typically 10,000 to 20,000 fluorescence "units", with about 75% emanating from the fluorometer background and the remainder from the MB probes. The plates were then placed in a thermal cycler for asymmetric amplification at the following temperatures: 94° for one minute; 10-15 cycles of 94° for 15 sec, 55° for 15 sec., 70° for 15 seconds; 60° for five minutes. The plates were then incubated at room temperature for at least 20 minutes and fluorescence measured as described above. The fluorescence readings obtained were stable for several hours. Specific fluorescence was defined as the difference in fluorescence before and after the asymmetric amplification. RED/GREEN ratios were defined as the specific fluorescence of MB-RED divided by that of MB-GREEN. RED/GREEN ratios were normalized to the ratio exhibited by the positive controls (25 genome equivalents of DNA from normal cells, as defined in Materials and Methods). We found that the ability of MB probes to discriminate between WT and mutant sequences under our conditions could not be reliably determined from experiments in which they were tested by hybridization to relatively short complementary single stranded oligonucleotides, and that actual PCR products had to be used for validation.

EXAMPLE 3

```
Oligonucleotides and DNA sequencing.
(SEQ ID NO: 1)
Primer F1:
5'-CATGTTCTAATATAGTCACATTTTCA-3';

(SEQ ID NO: 2)
Primer R1:
5'-TCTGAATTAGCTGTATCGTCAAGG-3';

(SEQ ID NO: 3)
Primer INT:
5'-TAGCTGTATCGTCAAGGCAC-3';

(SEQ ID NO: 4)
MB-RED:
5'-Cy3-CACGGGCCTGCTGAAAATGACTGCGTG-Dabcyl-3';

(SEQ ID NO: 5)
MB-GREEN:
5'-Fluorescein-CACGGGAGCTGGTGGCGTAGCGTG-Dabcyl-3'.
```

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.

EXAMPLE 4

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

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

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

Practical Considerations.

Numerous conditions were optimized to define conditions that could be reproducibly and generally applied. As outlined in FIG. 1A, the first step involves amplification from single template molecules. Most protocols for amplification from small numbers of template molecules use a nesting procedure, wherein a product resulting from one set of primers is used as template in a second reaction employing internal primers. As many applications of digital amplification are expected to require hundreds or thousands of separate amplifications, such nesting would be inconvenient and could lead to contamination problems. Hence, conditions were sought that would achieve robust amplification without nesting. The most important of these conditions involved the use of a polymerase that was activated only after heating and optimized concentrations of dNTP's, primers, buffer components, and temperature. The conditions specified in Examples 1-3 were defined after individually optimizing each of these components and proved suitable for amplification of several different human genomic DNA sequences. Though the time required for PCR was not particularly long (~2.5 hr), the number of cycles used was high and excessive compared to the number of cycles required to amplify the "average" single template molecule. The large cycle number was necessary because the template in some wells might not begin to be amplified until several PCR cycles had been completed. The large number of cycles ensured that every well (not simply the average well) would generate a substantial and roughly equal amount of PCR product if a template molecule were present within it.

The second step in Fig 1A involves the detection of these PCR products. It was necessary to considerably modify the standard MB probe approach in order for it to function efficiently in Digital Amplification applications. Theoretically, one separate MB probe could be used to detect each specific mutation that might occur within the queried sequence. By inclusion of one MB corresponding to WT sequence and another corresponding to mutant sequence, the nature of the PCR product would be revealed. Though this strategy could obviously be used effectively in some situations, it becomes complex when several different mutations are expected to occur within the same queried sequence. For example, in the c-Ki-Ras gene example explored here, twelve different base substitutions resulting in missense mutations could theoretically occur within codons 12 and 13, and at least seven of these are observed in naturally-occurring human cancers. To detect all twelve mutations as well as the WT sequence with individual Molecular Beacons would require 13 different probes. Inclusion of such a large number of MB probes would not only raise the background fluorescence but would be expensive. We therefore attempted to develop a single probe that would react with WT sequences better than any mutant sequence within the queried sequence. We found that the length of the loop sequence, its melting temperature, and the length and sequence of the stem were each important in determining the efficacy of such probes. Loops ranging from 14 to 26 bases and stems ranging from 4 to 6 bases, as well as numerous sequence variations of both stems and loops, were tested during the optimization procedure. For discrimination between WT and mutant sequences (MB-GREEN probe), we found that a **16** base pair loop, of melting temperature (Tm) 50-51=° , and a 4 bp by stem, of sequence 5'-CACG-3', were optimal. For MB-RED probes, the same stem, with a 19-20 by 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 discrininate 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 preformed 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).

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The invention claimed is:

1. A method for determining an allelic imbalance in 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 a selected genetic sequence on a first chromosome and a second number of assay samples which contain a reference genetic sequence on a second chromosome, wherein between 0.1 and 0.9 of the assay samples yield an amplification product;

comparing the first number of assay samples to the second number of assay samples to ascertain an allelic imbalance in the biological sample.

2. The method of claim 1 wherein the step of amplifying employs real-time polymerase chain reactions.

3. The method of claim 2 wherein the real-time polymerase chain reactions comprise a dual-labeled fluorogenic probe.

4. The method of claim 1 wherein the selected genetic sequence and the reference genetic sequence are non-polymorphic markers.

5. The method of claim 1 wherein the biological sample is from blood.

6. The method of claim 1 wherein the selected genetic sequence is a non-polymorphic marker.

7. The method of claim 1 wherein the reference genetic sequence is a non-polymorphic marker.

8. The method of claim 1 wherein between 0.1 and 0.6 of the assay samples yield an amplification product.

9. The method of claim 1 wherein between 0.3 and 0.5 of the assay samples yield an amplification product.

10. The method of claim 1 wherein between 0.1 and 0.9 of the assay samples yield an amplification product as determined by amplification of the selected genetic sequence.

11. The method of claim 1 wherein between 0.1 and 0.9 of the assay samples yield an amplification product as determined by amplification of the reference genetic sequence.

12. The method of claim 1 wherein between 0.1 and 0.6 of the assay samples yield an amplification product as determined by amplification of the selected genetic sequence.

13. The method of claim 1 wherein between 0.1 and 0.6 of the assay samples yield an amplification product as determined by amplification of the reference genetic sequence.

14. The method of claim 1 wherein between 0.3 and 0.5 of the assay samples yield an amplification product as determined by amplification of the selected genetic sequence.

15. The method of claim 1 wherein between 0.3 and 0.5 of the assay samples yield an amplification product as determined by amplification of the reference genetic sequence.

16. The method of claim 1 wherein the set comprises at least 500 assay samples.

17. The method of claim 1 wherein the set comprises at least 1000 assay samples.

18. The method of claim 1 wherein the amplified molecules in each of the assay samples in the first and second numbers of assay samples are homogeneous such that the first number of assay samples do not contain the reference genetic sequence and the second number of assay samples do not contain the selected genetic sequence.

19. A method for determining an allelic imbalance in a biological sample, comprising the steps of:

distributing nucleic acid template molecules from 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 a selected genetic sequence on a first chromosome and a second number of assay samples which contain a reference genetic sequence on a second chromosome;

comparing the first number of assay samples to the second number of assay samples to ascertain an allelic imbalance between the first chromosome and the second chromosome in the biological sample.

20. The method of claim 19 wherein between 0.1 and 0.9 of the assay samples yield an amplification product.

21. The method of claim 20 wherein between 0.1 and 0.9 of the assay samples yield a homogeneous amplification product.

22. The method of claim 19 wherein the biological sample is blood.

* * * * *

PTO/SB/26 (12-07)
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Under the Paperwork Reduction Act of 1995, no persons are required to respond to a collection of information unless it displays a valid OMB control number.

TERMINAL DISCLAIMER TO OBVIATE A DOUBLE PATENTING REJECTION OVER A "PRIOR" PATENT	Docket Number (Optional) 001107.00638

In re Application of: VOGELSTEIN ET AL.

Application No.: 11709742

Filed: 23 February 2007

For: DIGITAL AMPLIFICATION

The owner*, The Johns Hopkins University, of 100 percent interest in the instant application hereby disclaims, except as provided below, the terminal part of the statutory term of any patent granted on the instant application which would extend beyond the expiration date of the full statutory term **prior patent** No. U.S. 6,440,706 as the term of said prior patent is defined in 35 U.S.C. 154 and 173, and as the term of said **prior patent** is presently shortened by any terminal disclaimer. The owner hereby agrees that any patent so granted on the instant application shall be enforceable only for and during such period that it and the **prior patent** are commonly owned. This agreement runs with any patent granted on the instant application and is binding upon the grantee, its successors or assigns.

In making the above disclaimer, the owner does not disclaim the terminal part of the term of any patent granted on the instant application that would extend to the expiration date of the full statutory term as defined in 35 U.S.C. 154 and 173 of the **prior patent**, "as the term of said **prior patent** is presently shortened by any terminal disclaimer," in the event that said **prior patent** later:

expires for failure to pay a maintenance fee;
is held unenforceable;
is found invalid by a court of competent jurisdiction;
is statutorily disclaimed in whole or terminally disclaimed under 37 CFR 1.321;
has all claims canceled by a reexamination certificate;
is reissued; or
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Check either box 1 or 2 below, if appropriate.

1. ☐ For submissions on behalf of a business/organization (e.g., corporation, partnership, university, government agency, etc.), the undersigned is empowered to act on behalf of the business/organization.

I hereby declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code and that such willful false statements may jeopardize the validity of the application or any patent issued thereon.

2. ☑ The undersigned is an attorney or agent of record. Reg. No. 32,141

/Sarah A. Kagan/ — Signature
12 March 2010 — Date

Sarah A. Kagan — Typed or printed name

202 824 3000 — Telephone Number

☑ Terminal disclaimer fee under 37 CFR 1.20(d) included.

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.

*Statement under 37 CFR 3.73(b) is required if terminal disclaimer is signed by the assignee (owner).
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EXHIBIT 3

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APPLICATION NUMBER	PATENT NUMBER	GROUP ART UNIT	FILE WRAPPER LOCATION
11/709,742	7824889	1637	9200

OC000000050585403

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

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APPLICATION NO.	ISSUE DATE	PATENT NO.	ATTORNEY DOCKET NO.	CONFIRMATION NO.
11/709,742	11/02/2010	7824889	001107.00638	3875

22907 7590 10/13/2010

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

ISSUE NOTIFICATION

The projected patent number and issue date are specified above.

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

The Patent Term Adjustment is 659 day(s). Any patent to issue from the above-identified application will include an indication of the adjustment on the front page.

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

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

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

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

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

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Alexandria, Virginia 22313-1450
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Bib Data Sheet

CONFIRMATION NO. 3875

SERIAL NUMBER	FILING OR 371(c) DATE	CLASS	GROUP ART UNIT	ATTORNEY DOCKET NO.
11/709,742	02/23/2007 RULE	435	1637	001107.00638

APPLICANTS

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

**** CONTINUING DATA ***************************

This application is a CON of 10/828,295 04/21/2004 ABN
which is a DIV of 09/981,356 10/12/2001 PAT 6,753,147
which is a CON of 09/613,826 07/11/2000 PAT 6,440,706
which claims benefit of 60/146,792 08/02/1999

**** FOREIGN APPLICATIONS *********************

IF REQUIRED, FOREIGN FILING LICENSE GRANTED
** 03/26/2008

Foreign Priority claimed	35 USC 119 (a-d) conditions met	Verified and Acknowledged	STATE OR COUNTRY	SHEETS DRAWING	TOTAL CLAIMS	INDEPENDENT CLAIMS
☐ yes ☐ no	☐ yes ☐ no ☐ Met after Allowance	Examiner's Signature Initials	MD	7	48	5

ADDRESS
22907

TITLE

DIGITAL AMPLIFICATION

FILING FEE RECEIVED	FEES: Authority has been given in Paper
1905	No. _____ to charge/credit DEPOSIT ACCOUNT No. _____ for following:

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- ☐ 1.17 Fees (Processing Ext. of time)
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22907 7590 07/27/2010

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

APPLICATION NO.	FILING DATE	FIRST NAMED INVENTOR	ATTORNEY DOCKET NO.	CONFIRMATION NO.
11/709,742	02/23/2007	Bert Vogelstein	001107.00638	3875

TITLE OF INVENTION: DIGITAL AMPLIFICATION

APPLN. TYPE	SMALL ENTITY	ISSUE FEE DUE	PUBLICATION FEE DUE	PREV. PAID ISSUE FEE	TOTAL FEE(S) DUE	DATE DUE
nonprovisional	YES	~~$755~~ $1510	$300	$0	~~$1055~~ $1810	10/27/2010

EXAMINER	ART UNIT	CLASS-SUBCLASS
WOOLWINE, SAMUEL C	1637	435-091200

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

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

1 Banner & Witcoff, Ltd.
2
3

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

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

(A) NAME OF ASSIGNEE: The Johns Hopkins University

(B) RESIDENCE: (CITY and STATE OR COUNTRY): Baltimore, MD

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

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

4b. Payment of Fee(s): **(Please first reapply any previously paid issue fee shown above)**
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☐ Payment by credit card. Form PTO-2038 is attached.
☒ The Director is hereby authorized to charge the required fee(s), any deficiency, or credit any overpayment, to Deposit Account Number 190733 (enclose an extra copy of this form).

5. **Change in Entity Status** (from status indicated above)
☐ a. Applicant claims SMALL ENTITY status. See 37 CFR 1.27.
☒ b. Applicant is no longer claiming SMALL ENTITY status. See 37 CFR 1.27(g)(2).

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

Authorized Signature /Sarah A. Kagan/ Date 23 September 2010

Typed or printed name Sarah A. Kagan Registration No. 32141

Electronic Patent Application Fee Transmittal

Application Number:	11709742
Filing Date:	23-Feb-2007
Title of Invention:	DIGITAL AMPLIFICATION
First Named Inventor/Applicant Name:	Bert Vogelstein
Filer:	Sarah Anne Kagan.
Attorney Docket Number:	001107.00638

Filed as Large Entity

Utility under 35 USC 111(a) Filing Fees

Description	Fee Code	Quantity	Amount	Sub-Total in USD($)
Basic Filing:				
Pages:				
Claims:				
Miscellaneous-Filing:				
Petition:				
Patent-Appeals-and-Interference:				
Post-Allowance-and-Post-Issuance:				
Utility Appl issue fee	1501	1	1510	1510
Publ. Fee- early, voluntary, or normal	1504	1	300	300

Description	Fee Code	Quantity	Amount	Sub-Total in USD($)
Extension-of-Time:				
Miscellaneous:				
			Total in USD ($)	**1810**

Electronic Acknowledgement Receipt

EFS ID:	8479065
Application Number:	11709742
International Application Number:	
Confirmation Number:	3875
Title of Invention:	DIGITAL AMPLIFICATION
First Named Inventor/Applicant Name:	Bert Vogelstein
Customer Number:	22907
Filer:	Sarah Anne Kagan.
Filer Authorized By:	
Attorney Docket Number:	001107.00638
Receipt Date:	23-SEP-2010
Filing Date:	23-FEB-2007
Time Stamp:	11:09:32
Application Type:	Utility under 35 USC 111(a)

Payment information:

Submitted with Payment	yes
Payment Type	Deposit Account
Payment was successfully received in RAM	$1810
RAM confirmation Number	9444
Deposit Account	190733
Authorized User	

File Listing:

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

1	Issue Fee Payment (PTO-85B)	00638IFpayment.pdf	104832 77f975efa5643517cc08423f5ffe8b875122e304	no	1
Warnings:					
Information:					
2	Fee Worksheet (PTO-875)	fee-info.pdf	31882 aa2c24956faf6334397affb79a292c5267132a17	no	2
Warnings:					
Information:					
			Total Files Size (in bytes):	136714	

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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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22907 7590 07/27/2010

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SUITE 1200
WASHINGTON, DC 20005-4051

EXAMINER	
WOOLWINE, SAMUEL C	
ART UNIT	PAPER NUMBER
1637	

DATE MAILED: 07/27/2010

APPLICATION NO.	FILING DATE	FIRST NAMED INVENTOR	ATTORNEY DOCKET NO.	CONFIRMATION NO.
11/709,742	02/23/2007	Bert Vogelstein	001107.00638	3875

TITLE OF INVENTION: DIGITAL AMPLIFICATION

APPLN. TYPE	SMALL ENTITY	ISSUE FEE DUE	PUBLICATION FEE DUE	PREV. PAID ISSUE FEE	TOTAL FEE(S) DUE	DATE DUE
nonprovisional	YES	$755	$300	$0	$1055	10/27/2010

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

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

HOW TO REPLY TO THIS NOTICE:

I. Review the SMALL ENTITY status shown above.

If the SMALL ENTITY is shown as YES, verify your current SMALL ENTITY status:

A. If the status is the same, pay the TOTAL FEE(S) DUE shown above.

B. If the status above is to be removed, check box 5b on Part B - Fee(s) Transmittal and pay the PUBLICATION FEE (if required) and twice the amount of the ISSUE FEE shown above, or

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 box 5a on Part B - Fee(s) Transmittal and pay the PUBLICATION FEE (if required) and 1/2 the ISSUE FEE shown above.

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

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

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

PART B - FEE(S) TRANSMITTAL

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

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

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

22907 7590 07/27/2010

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

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

Certificate of Mailing or Transmission

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

(Depositor's name)

(Signature)

(Date)

APPLICATION NO.	FILING DATE	FIRST NAMED INVENTOR	ATTORNEY DOCKET NO.	CONFIRMATION NO.
11/709,742	02/23/2007	Bert Vogelstein	001107.00638	3875

TITLE OF INVENTION: DIGITAL AMPLIFICATION

APPLN. TYPE	SMALL ENTITY	ISSUE FEE DUE	PUBLICATION FEE DUE	PREV. PAID ISSUE FEE	TOTAL FEE(S) DUE	DATE DUE
nonprovisional	YES	$755	$300	$0	$1055	10/27/2010

EXAMINER	ART UNIT	CLASS-SUBCLASS
WOOLWINE, SAMUEL C	1637	435-091200

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

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

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

2. For printing on the patent front page, list

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

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

1 _____

2 _____

3 _____

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

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

(A) NAME OF ASSIGNEE (B) RESIDENCE: (CITY and STATE OR COUNTRY)

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

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

❑ Issue Fee

❑ Publication Fee (No small entity discount permitted)

❑ Advance Order - # of Copies _____

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

❑ A check is enclosed.

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

❑ The Director is hereby authorized to charge the required fee(s), any deficiency, or credit any overpayment, to Deposit Account Number _____ (enclose an extra copy of this form).

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

❑ a. Applicant claims SMALL ENTITY status. See 37 CFR 1.27.

❑ b. Applicant is no longer claiming SMALL ENTITY status. See 37 CFR 1.27(g)(2).

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

Authorized Signature _____ Date _____

Typed or printed name _____ Registration No. _____

This collection of information is required by 37 CFR 1.311. The information is required to obtain or retain a benefit by the public which is to file (and by the USPTO to process) an application. Confidentiality is governed by 35 U.S.C. 122 and 37 CFR 1.14. This collection is estimated to take 12 minutes to complete, including gathering, preparing, and submitting the completed application form to the USPTO. Time will vary depending upon the individual case. Any comments on the amount of time you require to complete this form and/or suggestions for reducing this burden, should be sent to the Chief Information Officer, U.S. Patent and Trademark Office, U.S. Department of Commerce, P.O. Box 1450, Alexandria, Virginia 22313-1450. DO NOT SEND FEES OR COMPLETED FORMS TO THIS ADDRESS. SEND TO: Commissioner for Patents, P.O. Box 1450, Alexandria, Virginia 22313-1450.

Under the Paperwork Reduction Act of 1995, no persons are required to respond to a collection of information unless it displays a valid OMB control number.

UNITED STATES PATENT AND TRADEMARK OFFICE

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

APPLICATION NO.	FILING DATE	FIRST NAMED INVENTOR	ATTORNEY DOCKET NO.	CONFIRMATION NO.
11/709,742	02/23/2007	Bert Vogelstein	001107.00638	3875

22907 7590 07/27/2010

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

EXAMINER	
WOOLWINE, SAMUEL C	
ART UNIT	PAPER NUMBER
1637	

DATE MAILED: 07/27/2010

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 407 day(s). 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 Patent Term Adjustment will be 407 day(s).

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

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

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

Notice of Allowability	Application No.	Applicant(s)	
	11/709,742	VOGELSTEIN ET AL.	
	Examiner	Art Unit	
	SAMUEL C. WOOLWINE	1637	

-- 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 *Applicant responses filed 07/12/2010 and 07/13/2010*.

2. ☒ The allowed claim(s) is/are *39-41,43,48-54,57-63,65-67 and 69*.

3. ☐ 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: _____.

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.

4. ☐ 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.

5. ☐ CORRECTED DRAWINGS (as "replacement sheets") must be submitted.
   (a) ☐ including changes required by the Notice of Draftsperson's Patent Drawing Review (PTO-948) attached
      1) ☐ hereto or 2) ☐ to Paper No./Mail Date _____.
   (b) ☐ including changes required by the attached Examiner's Amendment / Comment or in the Office action of Paper No./Mail Date _____.

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

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

Attachment(s)

1. ☐ Notice of References Cited (PTO-892)
2. ☐ Notice of Draftperson's Patent Drawing Review (PTO-948)
3. ☒ Information Disclosure Statements (PTO/SB/08), Paper No./Mail Date 06/25/2010
4. ☐ Examiner's Comment Regarding Requirement for Deposit of Biological Material
5. ☐ Notice of Informal Patent Application
6. ☐ Interview Summary (PTO-413), Paper No./Mail Date _____ .
7. ☒ Examiner's Amendment/Comment
8. ☐ Examiner's Statement of Reasons for Allowance
9. ☐ Other _____.

/Samuel Woolwine/
Primary Examiner, AU 1637

ALLOWANCE

The rejection under 35 USC 112, 1st paragraph made in the Office action mailed 06/11/2010 is moot per the cancellation of the affected claims and amendment of remaining claims to correct claim dependency.

The objection to the drawings/specification is withdrawn in view of Applicant's amendment to the specification submitted 07/12/2010. Applicant's supplemental amendment to Table 1 submitted 07/13/2010 is noted.

Claims 39-41, 43, 48-54, 57-63, 65-67 and 69 are allowed.

Any inquiry concerning this communication or earlier communications from the examiner should be directed to SAMUEL C. 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, AU1637

Issue Classification

Application/Control No.	Applicant(s)/Patent Under Reexamination
11709742	VOGELSTEIN ET AL.
Examiner	**Art Unit**
SAMUEL C WOOLWINE	1637

ORIGINAL		INTERNATIONAL CLASSIFICATION									
CLASS	SUBCLASS	CLAIMED					NON-CLAIMED				
435	91.2	C	1	2	P	19 / 34 (2006.01.01)	C	0	7	H	21 / 04 (2006.01.01)

CROSS REFERENCE(S)					
CLASS	SUBCLASS (ONE SUBCLASS PER BLOCK)				
536	24.31	24.33			

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

Final	Original	Final	Original	Final	Original	Final	Original	Final	Original	Final	Original	Final	Original	Final	Original
	1		17		33	6	49	19	65						
	2		18		34	7	50	20	66						
	3		19		35	8	51	21	67						
	4		20		36	9	52		68						
	5		21		37	10	53	22	69						
	6		22		38	11	54								
	7		23	1	39		55								
	8		24	2	40		56								
	9		25	3	41	12	57								
	10		26		42	13	58								
	11		27	4	43	14	59								
	12		28		44	15	60								
	13		29		45	16	61								
	14		30		46	17	62								
	15		31		47	18	63								
	16		32	5	48		64								

(Assistant Examiner)	(Date)	Total Claims Allowed: 22	
/Samuel Woolwine/ AU 1637	07/15/2010	O.G. Print Claim(s)	O.G. Print Figure
(Primary Examiner)	(Date)	1	1A

EAST Search History

EAST Search History (Interference)

Ref #	Hits	Search Query	DBs	Default Operator	Plurals	Time Stamp
L1	0	((first different separate) adj2 chromosome$1).clm. and (amplify amplifying amplified amplification).clm. and (allel$2 near3 imbalance).clm.	US-PGPUB; USPAT; UPAD	OR	OFF	2010/07/15 18:07
L2	1	"Term Removed" and imbalance	US-PGPUB; USPAT; UPAD	OR	OFF	2010/07/15 18:08
L3	6	number.clm. and (amplify amplifying amplified amplification).clm. and (allel$2 near3 imbalance).clm.	US-PGPUB; USPAT; UPAD	OR	OFF	2010/07/15 18:09

7/15/2010 6:11:51 PM

PTO/SB/08a (01-10)
Approved for use through 07/31/2012. OMB 0651-0031
U.S. Patent and Trademark Office; U.S. DEPARTMENT OF COMMERCE

INFORMATION DISCLOSURE STATEMENT BY APPLICANT

(Not for submission under 37 CFR 1.99)

Application Number	11709742
Filing Date	2007-02-23
First Named Inventor	Bert VOGELSTEIN, et al.
Art Unit	1637
Examiner Name	Woolwine, Samuel C
Attorney Docket Number	001107.00638

U.S.PATENTS Remove

Examiner Initial*	Cite No	Patent Number	Kind Code[1]	Issue Date	Name of Patentee or Applicant of cited Document	Pages,Columns,Lines where Relevant Passages or Relevant Figures Appear
	1					

If you wish to add additional U.S. Patent citation information please click the Add button. Add

U.S.PATENT APPLICATION PUBLICATIONS Remove

Examiner Initial*	Cite No	Publication Number	Kind Code[1]	Publication Date	Name of Patentee or Applicant of cited Document	Pages,Columns,Lines where Relevant Passages or Relevant Figures Appear
	1					

If you wish to add additional U.S. Published Application citation information please click the Add button. Add

FOREIGN PATENT DOCUMENTS Remove

Examiner Initial*	Cite No	Foreign Document Number[3]	Country Code[2] i	Kind Code[4]	Publication Date	Name of Patentee or Applicant of cited Document	Pages,Columns,Lines where Relevant Passages or Relevant Figures Appear	T[5]
	1							☐

If you wish to add additional Foreign Patent Document citation information please click the Add button Add

NON-PATENT LITERATURE DOCUMENTS Remove

Examiner Initials*	Cite No	Include name of the author (in CAPITAL LETTERS), title of the article (when appropriate), title of the item (book, magazine, journal, serial, symposium, catalog, etc), date, pages(s), volume-issue number(s), publisher, city and/or country where published.	T[5]

INFORMATION DISCLOSURE STATEMENT BY APPLICANT (Not for submission under 37 CFR 1.99)		
	Application Number	11709742
	Filing Date	2007-02-23
	First Named Inventor	Bert VOGELSTEIN, et al.
	Art Unit	1637
	Examiner Name	Woolwine, Samuel C
	Attorney Docket Number	001107.00638

/S.W./	1	Notice of Reasons for Rejection dispatched April 28, 2010 in Japanese Application No. 2001-513641 and English translation thereof.	☐
/S.W./	2	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).	☐
	3	~~Ruano, G. et al., "Haplotype of Multiple Polymorphisms Resolved by Enzymatic Amplification of Single DNA Molecules, " Proc. Nat. Acad. Science USA, 1990, pp. 6296-6300~~	☐

If you wish to add additional non-patent literature document citation information please click the Add button Add

EXAMINER SIGNATURE

Examiner Signature	/Samuel Woolwine/	Date Considered	07/15/2010

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

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

The Ruano reference is lined through because it has already been considered on a previous IDS.
/SW/

Search Notes	Application/Control No.	Applicant(s)/Patent Under Reexamination
	11709742	VOGELSTEIN ET AL.
	Examiner	Art Unit
	SAMUEL C WOOLWINE	1637

SEARCHED

Class	Subclass	Date	Examiner

SEARCH NOTES

Search Notes	Date	Examiner
Prosecution history of parent applications, keyword search in EAST (see printouts)	12/22/2009	SCW
Update search: keyword search in EAST (see printouts)	06/07/2010	SCW

INTERFERENCE SEARCH

Class	Subclass	Date	Examiner
	Keyword search in EAST (see printouts)	07/15/2010	SCW

PATENT

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re Application of	)	Group Art Unit: 1637
	)	
Bert VOGELSTEIN et al	)	Examiner: Woolwine, Samuel C.
	)	
Serial No. 11/709,742	)	Confirmation No. 3875
	)	
Filed: February 23, 2007	)	Atty. Dkt. No. 001107.00638
	)	
For: DIGITAL AMPLIFICATION	)	

SUPPLEMENTAL AMENDMENT

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

Sir:

This amendment supplements the amendment filed yesterday, July 12, 2010.

No fees are believed necessary. However, the U.S. Patent and Trademark Office is authorized to charge any necessary fees to our deposit account no. 19-0733.

- Amendments to the Specification begin on page 2 of this paper.
- Remarks begin on page 3 of this paper.

IN THE SPECIFICATION

Applicants respectfully request that the following Table 1 be substituted for that currently of record.

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 mutant alleles ~~mutations on one allele~~ vs. ~~one of the two mutations in each of two alleles~~ both mutations in the same allele	first mutation	second mutation
Allelic Imbalance	Quantitative analysis with non-polymorphic markers	marker sequence	marker from another chromosome

Remarks

Amendments

The amendment to the table is simply for increased clarity and is still supported at pages 8-9.

Respectfully submitted,

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

Date: July 13, 2010

Banner & Witcoff, Ltd.
Customer No. 22907

Electronic Acknowledgement Receipt

EFS ID:	8002558
Application Number:	11709742
International Application Number:	
Confirmation Number:	3875
Title of Invention:	Digital amplification
First Named Inventor/Applicant Name:	Bert Vogelstein
Customer Number:	22907
Filer:	Sarah Anne Kagan.
Filer Authorized By:	
Attorney Docket Number:	001107.00638
Receipt Date:	13-JUL-2010
Filing Date:	23-FEB-2007
Time Stamp:	12:51:14
Application Type:	Utility under 35 USC 111(a)

Payment information:

Submitted with Payment	no

File Listing:

Document Number	Document Description	File Name	File Size(Bytes)/ Message Digest	Multi Part /.zip	Pages (if appl.)
1	Supplemental Response or Supplemental Amendment	00638supp.pdf	90546 f17fd5a4e42bf7ab4f3546757e692a97756c8a21	no	3

Warnings:	
Information:	

Total Files Size (in bytes):	90546

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

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

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

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

PATENT

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re Application of	)	Group Art Unit: 1637
	)	
Bert VOGELSTEIN et al	)	Examiner: Woolwine, Samuel C.
	)	
Serial No. 11/709,742	)	Confirmation No. 3875
	)	
Filed: February 23, 2007	)	Atty. Dkt. No. 001107.00638
	)	
For: DIGITAL AMPLIFICATION	)	

RESPONSE AND AMENDMENT

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

Sir:

Please consider the amendment and remarks responsive to the non-final office action mailed June 11, 2010. Please charge any necessary fees to our deposit account no. 19-0733.

- Amendments to the Specification begin on page 2 of this paper.
- Amendments to the claims begin on page 6 of this paper.
- Remarks begin on page 11 of this paper.

IN THE SPECIFICATION

Applicants respectfully request that the following Table 1 be substituted for that currently of record.

Table 1. Potential Applications of Dig-PCR

Application	Example	Probe 1 Detects:	Probe 2 Detects:
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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~~ mutations on one allele vs. one of the two mutations in each of two alleles	first mutation	second mutation
Allelic Imbalance	Quantitative analysis with non-polymorphic markers	marker sequence	marker from another chromosome

Please replace the paragraph beginning on page 4, line 16:

FIGS. 1A, 1B, 1C. Schematic of experimental design. (Fig. 1A) The basic two steps involved: PCR on diluted DNA samples is followed by addition of fluorescent probes which discriminate between WT and mutant alleles and subsequent fluorometry. (Fig. 1B) Principle of molecular beacon analysis. In the stem-loop configuration, fluorescence from a dye at the 5' end of the oligonucleotide probe is quenched by a Dabcyl group at the 3' end. Upon hybridization to a template, the dye is separated from the quencher, resulting in increased fluorescence. Modified from Marras *et al.* (Fig. 1C) Oligonucleotide design. Primers F1 and R1 are used to amplify the

genomic region of interest. Primer INT is used to produce single stranded DNA from the original PCR products during a subsequent asymmetric PCR step (see Materials and Methods). MB-RED is a Molecular Beacon which detects any appropriate PCR product, whether it is WT or mutant at the queried codons. MB-GREEN is a Molecular Beacon which preferentially detects the WT PCR product.

Please replace the paragraph beginning page 5, line 3.

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).

Please replace the paragraph beginning page 5, line 24.

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.

Please replace the paragraph beginning page 6, line 5.

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.

Please replace the paragraph beginning on page 14, line 5.

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.

IN THE CLAIMS

Please substitute the following claim set for those currently or record:

1-38. (Cancelled)

39. (Previously Presented) A method for determining an allelic imbalance in 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 a selected genetic sequence on a first chromosome and a second number of assay samples which contain a reference genetic sequence on a second chromosome, wherein between 0.1 and 0.9 of the assay samples yield an amplification product;

comparing the first number of assay samples to the second number of assay samples to ascertain an allelic imbalance in the biological sample.

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

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

42. (Cancelled)

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

44. (Cancelled)

45. (Cancelled)

46. (Cancelled)

47. (Cancelled)

48. (Currently amended) The method of claim 39 ~~or 45~~ wherein the biological sample is from blood.

49. (Previously Presented) The method of claim 39 wherein the selected genetic sequence is a non-polymorphic marker.

50. (Previously Presented) The method of claim 39 wherein the reference genetic sequence is a non-polymorphic marker.

51. (Currently amended) The method of claim 39 ~~or 45~~ wherein between 0.1 and 0.6 of the assay samples yield an amplification product.

52. (Currently amended) The method of claim 39 ~~or 45~~ wherein between 0.3 and 0.5 of the assay samples yield an amplification product.

53. (Previously Presented) The method of claim 39 wherein between 0.1 and 0.9 of the assay samples yield an amplification product as determined by amplification of the selected genetic sequence.

54. (Previously Presented) The method of claim 39 wherein between 0.1 and 0.9 of the assay samples yield an amplification product as determined by amplification of the reference genetic sequence.

55. (Cancelled)

56. (Cancelled)

57. (Previously Presented) The method of claim 39 wherein between 0.1 and 0.6 of the assay samples yield an amplification product as determined by amplification of the selected genetic sequence.

58. (Previously Presented) The method of claim 39 wherein between 0.1 and 0.6 of the assay samples yield an amplification product as determined by amplification of the reference genetic sequence.

59. (Previously Presented) The method of claim 39 wherein between 0.3 and 0.5 of the assay samples yield an amplification product as determined by amplification of the selected genetic sequence.

60. (Previously Presented) The method of claim 39 wherein between 0.3 and 0.5 of the assay samples yield an amplification product as determined by amplification of the reference genetic sequence.

61. (Currently amended) The method of claim 39 ~~or 45~~ wherein the set comprises at least 500 assay samples.

62. (Currently amended) The method of claim 39 ~~or 45~~ wherein the set comprises at least 1000 assay samples.

63. (Previously Presented) The method of claim 39 wherein the amplified molecules in each of the assay samples in the first and second numbers of assay samples are homogeneous such that the first number of assay samples do not contain the reference genetic sequence and the second number of assay samples do not contain the selected genetic sequence.

64. (Cancelled)

65. (Previously Presented) A method for determining an allelic imbalance in a biological sample, comprising the steps of:

distributing nucleic acid template molecules from 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 a selected genetic sequence on a first chromosome and a second number of assay samples which contain a reference genetic sequence on a second chromosome;

comparing the first number of assay samples to the second number of assay samples to ascertain an allelic imbalance between the first chromosome and the second chromosome in the biological sample.

66. (Previously Presented) The method of claim 65 wherein between 0.1 and 0.9 of the assay samples yield an amplification product.

67. (Previously Presented) The method of claim 66 wherein between 0.1 and 0.9 of the assay samples yield a homogeneous amplification product.

68. (Cancelled)

69. (Currently amended) The method of claim 65 ~~or 68~~ wherein the biological sample is blood.

Remarks

Amendments

The clarifying amendment to the table is supported at pages 8-9.

The specification has been amended to properly recite Figures 1A, 1B, 1C in the Brief Description of the Drawings.

The specification was further amended to reference the sequence listing for each disclosed sequence in Figures 2, 4, and 5 and in Example 3. The references for the Figures were inserted in the Brief Description of the Drawings.

New matter

Claims rejected for new matter are cancelled by the above amendment. Applicants do not, however, agree with the U.S. Patent and Trademark Office's position regarding the scope of the disclosure supporting the claims. In particular, the Table provides an example of various embodiments in a column headed "examples." The U.S. Patent and Trademark Office has erroneously interpreted the disclosed invention as limited to the examples provided. Applicants reserve the right to pursue the cancelled subject matter in other applications.

Respectfully submitted,

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

Date: July 12, 2010

Banner & Witcoff, Ltd.
Customer No. 22907

Electronic Acknowledgement Receipt

EFS ID:	7993863
Application Number:	11709742
International Application Number:	
Confirmation Number:	3875
Title of Invention:	Digital amplification
First Named Inventor/Applicant Name:	Bert Vogelstein
Customer Number:	22907
Filer:	Sarah Anne Kagan./Jennifer Brady
Filer Authorized By:	Sarah Anne Kagan.
Attorney Docket Number:	001107.00638
Receipt Date:	12-JUL-2010
Filing Date:	23-FEB-2007
Time Stamp:	14:19:40
Application Type:	Utility under 35 USC 111(a)

Payment information:

Submitted with Payment	no

File Listing:

Document Number	Document Description	File Name	File Size(Bytes)/ Message Digest	Multi Part /.zip	Pages (if appl.)
1		Amendment_NFOA_dtd_06_11_2010.pdf	115075 30728a21ad45f250ad5890ea60b7c515cdf0b08a	yes	11

	Multipart Description/PDF files in .zip description		
	Document Description	**Start**	**End**
	Amendment/Req. Reconsideration-After Non-Final Reject	1	1
	Specification	2	5
	Claims	6	10
	Applicant Arguments/Remarks Made in an Amendment	11	11
Warnings:			
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National Stage of an International Application under 35 U.S.C. 371
If a timely submission to enter the national stage of an international application is compliant with the conditions of 35 U.S.C. 371 and other applicable requirements a Form PCT/DO/EO/903 indicating acceptance of the application as a national stage submission under 35 U.S.C. 371 will be issued in addition to the Filing Receipt, in due course.

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

PTO/SB/08a (01-10)
Approved for use through 07/31/2012. OMB 0651-0031
U.S. Patent and Trademark Office; U.S. DEPARTMENT OF COMMERCE

INFORMATION DISCLOSURE STATEMENT BY APPLICANT

(Not for submission under 37 CFR 1.99)

Application Number	11709742
Filing Date	2007-02-23
First Named Inventor	Bert VOGELSTEIN, et al.
Art Unit	1637
Examiner Name	Woolwine, Samuel C
Attorney Docket Number	001107.00638

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INFORMATION DISCLOSURE STATEMENT BY APPLICANT (Not for submission under 37 CFR 1.99)		
	Application Number	11709742
	Filing Date	2007-02-23
	First Named Inventor	Bert VOGELSTEIN, et al.
	Art Unit	1637
	Examiner Name	Woolwine, Samuel C
	Attorney Docket Number	001107.00638

	1	Notice of Reasons for Rejection dispatched April 28, 2010 in Japanese Application No. 2001-513641 and English translation thereof.	☐
	2	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).	☐
	3	Ruano, G. et al., "Haploytype of Multiple Polymorphisms Resolved by Enzymatic Amplifciation of Single DNA M oecules, " Proc. Nat. Acad. Science USA, 1990, pp. 6296-6300	☐

If you wish to add additional non-patent literature document citation information please click the Add button Add

EXAMINER SIGNATURE

Examiner Signature		Date Considered	

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

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

INFORMATION DISCLOSURE STATEMENT BY APPLICANT (Not for submission under 37 CFR 1.99)		
	Application Number	11709742
	Filing Date	2007-02-23
	First Named Inventor	Bert VOGELSTEIN, et al.
	Art Unit	1637
	Examiner Name	Woolwine, Samuel C
	Attorney Docket Number	001107.00638

CERTIFICATION STATEMENT

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

☒ That each item of information contained in the information disclosure statement was first cited in any communication from a foreign patent office in a counterpart foreign application not more than three months prior to the filing of the information disclosure statement. See 37 CFR 1.97(e)(1).

OR

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

☐ See attached certification statement.

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

☐ None

SIGNATURE

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

Signature	/Sarah A. Kagan/	Date (YYYY-MM-DD)	2010-06-22
Name/Print	Sarah A. Kagan	Registration Number	32141

This collection of information is required by 37 CFR 1.97 and 1.98. The information is required to obtain or retain a benefit by the public which is to file (and by the USPTO to process) an application. Confidentiality is governed by 35 U.S.C. 122 and 37 CFR 1.14. This collection is estimated to take 1 hour to complete, including gathering, preparing and submitting the completed application form to the USPTO. Time will vary depending upon the individual case. Any comments on the amount of time you require to complete this form and/or suggestions for reducing this burden, should be sent to the Chief Information Officer, U.S. Patent and Trademark Office, U.S. Department of Commerce, P.O. Box 1450, Alexandria, VA 22313-1450. DO NOT SEND FEES OR COMPLETED FORMS TO THIS ADDRESS. **SEND TO: Commissioner for Patents, P.O. Box 1450, Alexandria, VA 22313-1450.**

Privacy Act Statement

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

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

1. The information on this form will be treated confidentially to the extent allowed under the Freedom of Information Act (5 U.S.C. 552) and the Privacy Act (5 U.S.C. 552a). Records from this system of records may be disclosed to the Department of Justice to determine whether the Freedom of Information Act requires disclosure of these record s.

2. A record from this system of records may be disclosed, as a routine use, in the course of presenting evidence to a court, magistrate, or administrative tribunal, including disclosures to opposing counsel in the course of settlement negotiations.

3. A record in this system of records may be disclosed, as a routine use, to a Member of Congress submitting a request involving an individual, to whom the record pertains, when the individual has requested assistance from the Member with respect to the subject matter of the record.

4. A record in this system of records may be disclosed, as a routine use, to a contractor of the Agency having need for the information in order to perform a contract. Recipients of information shall be required to comply with the requirements of the Privacy Act of 1974, as amended, pursuant to 5 U.S.C. 552a(m).

5. A record related to an International Application filed under the Patent Cooperation Treaty in this system of records may be disclosed, as a routine use, to the International Bureau of the World Intellectual Property Organization, pursuant to the Patent Cooperation Treaty.

6. A record in this system of records may be disclosed, as a routine use, to another federal agency for purposes of National Security review (35 U.S.C. 181) and for review pursuant to the Atomic Energy Act (42 U.S.C. 218(c)).

7. A record from this system of records may be disclosed, as a routine use, to the Administrator, General Services, or his/her designee, during an inspection of records conducted by GSA as part of that agency's responsibility to recommend improvements in records management practices and programs, under authority of 44 U.S.C. 2904 and 2906. Such disclosure shall be made in accordance with the GSA regulations governing inspection of records for this purpose, and any other relevant (i.e., GSA or Commerce) directive. Such disclosure shall not be used to make determinations about individuals.

8. A record from this system of records may be disclosed, as a routine use, to the public after either publication of the application pursuant to 35 U.S.C. 122(b) or issuance of a patent pursuant to 35 U.S.C. 151. Further, a record may be disclosed, subject to the limitations of 37 CFR 1.14, as a routine use, to the public if the record was filed in an application which became abandoned or in which the proceedings were terminated and which application is referenced by either a published application, an application open to public inspections or an issued patent.

9. A record from this system of records may be disclosed, as a routine use, to a Federal, State, or local law enforcement agency, if the USPTO becomes aware of a violation or potential violation of law or regulation.

Electronic Acknowledgement Receipt

EFS ID:	7897910
Application Number:	11709742
International Application Number:	
Confirmation Number:	3875
Title of Invention:	Digital amplification
First Named Inventor/Applicant Name:	Bert Vogelstein
Customer Number:	22907
Filer:	Sarah Anne Kagan./Jennifer Brady
Filer Authorized By:	Sarah Anne Kagan.
Attorney Docket Number:	001107.00638
Receipt Date:	25-JUN-2010
Filing Date:	23-FEB-2007
Time Stamp:	17:19:09
Application Type:	Utility under 35 USC 111(a)

Payment information:

Submitted with Payment	no

File Listing:

Document Number	Document Description	File Name	File Size(Bytes)/ Message Digest	Multi Part /.zip	Pages (if appl.)
1	Information Disclosure Statement (IDS) Filed (SB/08)	IDS_SB08_off_JPOA_dtd_04_28_2010.PDF	612431 1b6d7f0591a1deaed6c96876e648fd7c3bf5b70c	no	4

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2	NPL Documents	Notice_of_Reasons_for_Rejection_dtd_04_28_2010_JP2001-513641.PDF	222780 ea8cc419c94bbf201080ec2cfb009ca3de422506	no	6
Warnings:					
Information:					
3	NPL Documents	Stephens_et_al_TheoreticalUnderpinning_SingleMoleculeDilutionMethod_DirectHaplotypeResolution.PDF	1153082 f803d55f35cceb559ba91700b0f36a7d3655912e	no	7
Warnings:					
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New Applications Under 35 U.S.C. 111

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

National Stage of an International Application under 35 U.S.C. 371

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

New International Application Filed with the USPTO as a Receiving Office

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

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APPLICATION NO.	FILING DATE	FIRST NAMED INVENTOR	ATTORNEY DOCKET NO.	CONFIRMATION NO.
11/709,742	02/23/2007	Bert Vogelstein	001107.00638	3875

22907 7590 06/11/2010

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

EXAMINER	
WOOLWINE, SAMUEL C	
ART UNIT	**PAPER NUMBER**
1637	

MAIL DATE	DELIVERY MODE
06/11/2010	PAPER

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

The time period for reply, if any, is set in the attached communication.

Office Action Summary	Application No.	Applicant(s)	
	11/709,742	VOGELSTEIN ET AL.	
	Examiner	Art Unit	
	SAMUEL C. WOOLWINE	1637	

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

Period for Reply

A SHORTENED STATUTORY PERIOD FOR REPLY IS SET TO EXPIRE *3* MONTH(S) OR THIRTY (30) DAYS, WHICHEVER IS LONGER, FROM THE MAILING DATE OF THIS COMMUNICATION.

- Extensions of time may be available under the provisions of 37 CFR 1.136(a). In no event, however, may a reply be timely filed after SIX (6) MONTHS from the mailing date of this communication.
- If NO period for reply is specified above, the maximum statutory period will apply and will expire SIX (6) MONTHS from the mailing date of this communication.
- Failure to reply within the set or extended period for reply will, by statute, cause the application to become ABANDONED (35 U.S.C. § 133). Any reply received by the Office later than three months after the mailing date of this communication, even if timely filed, may reduce any earned patent term adjustment. See 37 CFR 1.704(b).

Status

1)☒ Responsive to communication(s) filed on *12 March 2010*.

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) *39-41,43 and 45-69* is/are pending in the application.

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

5)☒ Claim(s) *39-41,43,49,50,53,54,57-60,63 and 65-67* is/are allowed.

6)☒ Claim(s) *45-48,51,52,55,56,61,62,64,68 and 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 *18 June 2008* 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).

11)☐ 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

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

a)☐ All b)☐ Some * c)☐ None of:

1.☐ Certified copies of the priority documents have been received.

2.☐ Certified copies of the priority documents have been received in Application No. _____.

3.☐ Copies of the certified copies of the priority documents have been received in this National Stage application from the International Bureau (PCT Rule 17.2(a)).

* See the attached detailed Office action for a list of the certified copies not received.

Attachment(s)

1) ☐ Notice of References Cited (PTO-892)
2) ☐ Notice of Draftsperson's Patent Drawing Review (PTO-948)
3) ☒ Information Disclosure Statement(s) (PTO/SB/08) Paper No(s)/Mail Date *03/05/2010*.
4) ☐ Interview Summary (PTO-413) Paper No(s)/Mail Date. _____ .
5) ☐ Notice of Informal Patent Application
6) ☐ Other: _____.

DETAILED ACTION

Status

Applicant's response filed 03/12/2010 is acknowledged. In view of the terminal disclaimer filed 04/16/2010, the double-patenting rejection made in the Office action mailed 12/29/2009 is withdrawn.

The examiner has identified some new issues with regard to the application and the claims, and new objections and rejections are set forth below. Therefore, this Office action is NON-FINAL.

Claims 39-41, 43, 49, 50, 53, 54, 57-60, 63, 65-67 are allowed. Claims 48, 51, 52, 61, 62 and 69 would be allowable but for their partial dependence from rejected claims 45 and 68.

Specification & Drawings

Page 14 of the specification as filed displays nucleic acid sequences. Figures 2, 4 and 5 also display nucleic acid sequences.

As noted in MPEP 2422.01, any unbranched nucleic acid sequence having 10 nucleotides or more, and specifying at least 4 nucleotides (i.e. nucleotides other than "n"), fall within these definitions. In addition, MPEP 2422.02 states: "…when a sequence is presented in a drawing, regardless of the format or the manner of presentation of that sequence in the drawing, the sequence must still be included in the Sequence Listing and the sequence identifier (“SEQ ID NO:X”) must be used, either in the drawing or in the Brief Description of the Drawings."

In addition, 37 CFR 1.821(d) requires:

"Where the description or claims of a patent application discuss a sequence that is set forth in the "Sequence Listing" in accordance with paragraph (c) of this section, reference must be made to the sequence by use of the sequence identifier, preceded by "SEQ ID NO:" in the text of the description or claims, even if the sequence is also embedded in the text of the description or claims of the patent application."

Therefore, the specification and drawings are objected to until such amendments are made to include the appropriate SEQ ID NOs alongside the displayed nucleotides sequences.

Claim Rejections - 35 USC § 112

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 45-48, 51, 52, 55, 56, 61, 62, 64, 68 and 69 are rejected under 35 U.S.C. 112, first paragraph, as failing to comply with the written description requirement. The claim(s) contains subject matter which was not described in the specification in such a way as to reasonably convey to one skilled in the relevant art that the inventor(s), at the time the application was filed, had possession of the claimed invention. This is a NEW MATTER rejection.

Unless clearly stated otherwise, nothing in the examiner's explanation below should be construed as providing support for an amendment. Any amendments to the claims should be clearly supported by the disclosure as filed and so indicated by Applicant.

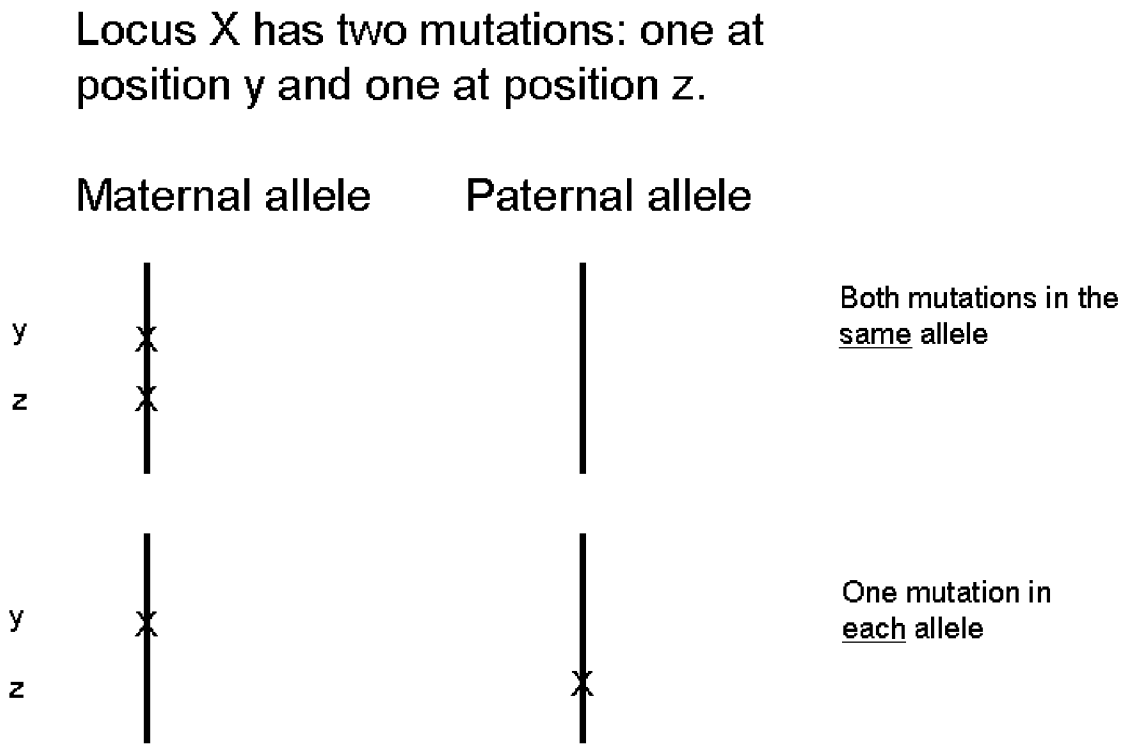
Independent claims 45 and 68 are drawn to methods "for determining an allelic imbalance" comprising determining "a first number of assay samples which contain a first allelic form of a marker and a second number of assay samples which contain a second allelic form of the marker". It is noted that claims 45 and 68 in their current form resulted from an amendment filed 06/30/2009. To support "an allelic imbalance" in claim 45, Applicant cites to Table 1, application #7 (see page 13 of the amendment filed 06/30/2009). To support "a first allelic form of a marker" in claim 45, Applicant cites to Table 1, application #6, in particular the term "allelic discrimination". It is respectfully asserted that: 1) Applicant is combining two separate applications of digital PCR, which combination does not appear in the disclosure as filed, and 2) application #6 from Table 1 does not determine an allelic imbalance. Moreover, there is no disclosure in the specification as filed for determining an allelic imbalance by measuring two different "allelic forms" of a marker. The only disclosure of determining an allelic imbalance is by assaying a first marker on one chromosome, and a second marker from another chromosome (see Table 1, application #7). This says nothing about two allelic forms of a single marker. In fact, Table 1 clearly indicates that the markers used to determine allelic imbalance are non-polymorphic. Hence there could be no "first allelic form" and "second allelic form", since this would mean the marker is polymorphic.

Based on page 3, paragraph 2 of the specification as filed, and Table 1, application #7, it is clear that the manner in which allelic imbalance is determined is as follows: the number of assay samples producing an amplification product for a non-polymorphic maker on one chromosome (e.g. chromosome 4) is compared to the

number of assay samples producing an amplification product for a non-polymorphic marker on another chromosome (e.g. chromosome 7). In this hypothetical example, if 20 out of 100 assay samples gave an amplicon for the marker on chromosome 4, but only 10 out of 100 assay samples gave an amplicon for the marker on chromosome 7, the conclusion would be that there is an imbalance between those markers (e.g. a deletion of one copy of chromosome 7 the portion thereof that contains the marker; or an extra copy of chromosome 4 or a duplication of a portion thereof that contained the marker).

This is entirely different than what is happening in Table 1, application #6. Incidentally, this is an appropriate place to point out that there appears to be an error in Table 1. A minimal explanation of this application of digital PCR is found beginning at the last full sentence of page 8 through the first full sentence of page 9 of the specification as filed. As stated there, one can use the method to determine "allelic status" where two mutations are present, by distinguishing whether one variant (mutation) is present in each allele (i.e. maternal and paternal allele) versus both mutations occurring in the same allele. Of course this only applies to diploid organisms. Note however that Table 1, application #6 ("Allelic discrimination") reads: "Two different alleles mutated vs. one mutation in each of two alleles." This statement is erroneous, because each option describes the same situation: if there is one mutation in each of two alleles, then two different alleles are mutated. It would appear that the statement in the table should read "Two different alleles mutated vs. both mutations in the same allele", or some similar language. Applicant is advised to amend Table 1 based on the

statement at pages 8-9 of the specification. The embodiment described as "Allelic discrimination" in Table 1, and discussed at pages 8-9 of the specification can be understood schematically as follows:



This is not what the disclosure as filed refers to as "Allelic imbalance". Rather, this is what the disclosure as filed refers to as "Allelic discrimination" (Table 1) or determining the "allelic status" (page 8, last full sentence). Moreover, it is not understood how *this* application of digital PCR would be achieved by comparing the number of assay samples positive for "a first allelic form of a marker" (or as more correctly stated in Table 1, a "first mutation") with the number of assay samples positive for "a second allelic form of a marker" (or as in Table 1, a "second mutation"). Indeed, one would expect the two numbers to be the same. That is, given the first scenario

(both mutations present in the same allele), one would expect the number of assay samples positive for the mutation at position y, and the number of assay samples positive for the mutation at position z, to be the same. The same is true of the second scenario (one mutation in each allele). What would *distinguish* the two situations is this: in the first scenario (both mutations in the same allele), the same assay samples positive for the mutation at position y would be positive for the mutation at position z (assuming that an individual nucleic acid molecule is not broken or sheared between positions y and z, which would be a function of the distance between y and z and the manner in which the nucleic acid is handled). However, in the second scenario (one mutation in each allele), while there would still be an equal number of assay samples positive for each mutation, one would expect that any individual assay sample is not positive for the mutation at y *and* positive for the mutation at z (this is assuming the sample was diluted to the extent that any individual assay sample contains no more than one copy of any individual nucleic acid target, which is the whole basis of digital PCR). Hence, the application wherein digital PCR is used for "Allelic discrimination" (Table 1) or determining "allelic status" (page 8, last full sentence) would not be based on comparing numbers of assay samples, but would instead rely on determining *which* samples were positive for a "first mutation" and a "second mutation". Unfortunately, the examiner does not see any disclosure of this procedure in the application as filed. Although the examiner has been able to determine how one *would* perform allelic discrimination using digital PCR, the application as filed does not disclose this.

Therefore, independent claims 45 and 68, and all claims dependent therefrom, are rejected as new matter, and the examiner is not able to recommend a manner of claiming embodiments drawn to "allelic discrimination" or determining "allelic status" as discussed above.

Conclusion

Any inquiry concerning this communication or earlier communications from the examiner should be directed to SAMUEL C. 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/
Examiner, Art Unit 1637

Search Notes	Application/Control No.	Applicant(s)/Patent Under Reexamination
	11709742	VOGELSTEIN ET AL.
	Examiner	Art Unit
	SAMUEL C WOOLWINE	1637

SEARCHED			
Class	Subclass	Date	Examiner

SEARCH NOTES		
Search Notes	Date	Examiner
Prosecution history of parent applications, keyword search in EAST (see printouts)	12/22/2009	SCW
Update search: keyword search in EAST (see printouts)	06/07/2010	SCW

INTERFERENCE SEARCH			
Class	Subclass	Date	Examiner

EAST Search History

EAST Search History (Prior Art)

Ref #	Hits	Search Query	DBs	Default Operator	Plurals	Time Stamp
L1	1	"6143496".pn.	USPAT	OR	OFF	2010/06/07 21:46
L2	2180	((sample specimen) near5 (dilut$3 split$4 divid$3)) same (pcr amplif$&)	US-PGPUB; USPAT; USOCR; EPO; JPO; DERWENT	OR	OFF	2010/06/07 22:20
L3	319	l2 and (@ad<"19990802" @pd<"19990802")	US-PGPUB; USPAT; USOCR; EPO; JPO; DERWENT	OR	OFF	2010/06/07 22:21
L4	142	l3 and ((count$3 number) with (positive amplicon$1 product$1))	US-PGPUB; USPAT; USOCR; EPO; JPO; DERWENT	OR	OFF	2010/06/07 22:22
L5	163	(allelic near2 (imbalance$1 ratio ratios)) and ((count$3 number) with (positive amplicon$1 product$1))	US-PGPUB; USPAT; USOCR; EPO; JPO; DERWENT	OR	OFF	2010/06/07 22:34
L6	34	(allelic near2 (imbalance$1 ratio ratios)) and ((count$3 number) with (positive amplicon$1 product$1) with (samples portions aliquots fractions))	US-PGPUB; USPAT; USOCR; EPO; JPO; DERWENT	OR	OFF	2010/06/07 22:35
L7	0	l6 and (@ad<"19990802" @pd<"19990802")	US-PGPUB; USPAT; USOCR; EPO; JPO; DERWENT	OR	OFF	2010/06/07 22:46
L8	2280	(single adj1 (molecule copy target nucleic)) near7 (amplification pcr)	US-PGPUB; USPAT; USOCR; EPO; JPO; DERWENT	OR	OFF	2010/06/07 22:48
L9	1801	(single adj1 (molecule copy target nucleic)) near5 (amplification pcr)	US-PGPUB; USPAT; USOCR; EPO; JPO; DERWENT	OR	OFF	2010/06/07 22:48
L10	264	l9 and (@ad<"19990802" @pd<"19990802")	US-PGPUB; USPAT; USOCR; EPO; JPO; DERWENT	OR	OFF	2010/06/07 22:49

L11	133	l10 and ((count$3 number) with (positive amplicon$1 product$1))	US-PGPUB; USPAT; USOCR; EPO; JPO; DERWENT	OR	OFF	2010/06/07 22:49
L12	72	l11 and (allelic allele alleles)	US-PGPUB; USPAT; USOCR; EPO; JPO; DERWENT	OR	OFF	2010/06/07 22:50
L13	44	l12 and ((count$3 number) near3(positive amplicon$1 product$1))	US-PGPUB; USPAT; USOCR; EPO; JPO; DERWENT	OR	OFF	2010/06/07 22:51
S1	1	"709742".ap. and kinzler.in.	US-PGPUB	OR	OFF	2010/06/07 17:15

6/7/2010 10:54:46 PM
C:\Documents and Settings\swoolwine\My Documents\EAST\Workspaces\11709742.wsp

PTO/SB/08a (01-10)
Approved for use through 07/31/2012. OMB 0651-0031
U.S. Patent and Trademark Office; U.S. DEPARTMENT OF COMMERCE

INFORMATION DISCLOSURE STATEMENT BY APPLICANT

(Not for submission under 37 CFR 1.99)

Application Number	11709742
Filing Date	2007-02-23
First Named Inventor	VOGELSTEIN, Bert
Art Unit	1637
Examiner Name	WOOLWINE, Samuel C.
Attorney Docket Number	001107.00638

U.S.PATENTS Remove

Examiner Initial*	Cite No	Patent Number	Kind Code[1]	Issue Date	Name of Patentee or Applicant of cited Document	Pages,Columns,Lines where Relevant Passages or Relevant Figures Appear
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Examiner Initials*	Cite No	Include name of the author (in CAPITAL LETTERS), title of the article (when appropriate), title of the item (book, magazine, journal, serial, symposium, catalog, etc), date, pages(s), volume-issue number(s), publisher, city and/or country where published.	T[5]

INFORMATION DISCLOSURE STATEMENT BY APPLICANT (Not for submission under 37 CFR 1.99)		
	Application Number	11709742
	Filing Date	2007-02-23
	First Named Inventor	VOGELSTEIN, Bert
	Art Unit	1637
	Examiner Name	WOOLWINE, Samuel C.
	Attorney Docket Number	001107.00638

/S.W./	1	Newton, PCR Essential Data, pages 51-52, 1995	☐

If you wish to add additional non-patent literature document citation information please click the Add button Add

EXAMINER SIGNATURE

Examiner Signature	/Samuel Woolwine/	Date Considered	06/07/2010

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

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

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APPLICATION NUMBER	FILING OR 371(C) DATE	FIRST NAMED APPLICANT	ATTY. DOCKET NO./TITLE
11/709,742	02/23/2007	Bert Vogelstein	001107.00638

CONFIRMATION NO. 3875

POA ACCEPTANCE LETTER

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

OC000000041164977

Date Mailed: 04/16/2010

NOTICE OF ACCEPTANCE OF POWER OF ATTORNEY

This is in response to the Power of Attorney filed 03/12/2010.

The Power of Attorney in this application is accepted. Correspondence in this application will be mailed to the above address as provided by 37 CFR 1.33.

/amwise/

Office of Data Management, Application Assistance Unit (571) 272-4000, or (571) 272-4200, or 1-888-786-0101

Application Number	Application/Control No.	Applicant(s)/Patent Under Reexamination
	11709742	VOGELSTEIN ET AL.

Document Code - DISQ	Internal Document – DO NOT MAIL

TERMINAL DISCLAIMER	☒ APPROVED	☐ DISAPPROVED
Date Filed: 03/12/2010	**This patent is subject to a Terminal Disclaimer**	

Approved/Disapproved by:	
APRIL M. WISE	

U.S. Patent and Trademark Office

PATENT

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re Application of	)	Group Art Unit: 1637
	)	
Bert VOGELSTEIN et al	)	Examiner: Samuel Woolwine
	)	
Serial No. 11/709,742	)	Confirmation No. 3875
	)	
Filed: February 23, 2007	)	Atty. Dkt. No. 001107.00638
	)	
For: DIGITAL AMPLIFICATION	)	

RESPONSE TO OFFICE ACTION

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

Sir:

In response to the Office Action mailed December 29, 2009, applicants submit a terminal disclaimer over the cited patent. It is respectfully submitted that this overcomes the double patenting rejection and puts the application in condition for allowance.

No extension of time fee is believed due in connection with this response. However, should the Patent and Trademark Office determine that any additional fee is required, please charge our Deposit Account No. 19-0733.

Respectfully submitted,

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

Date: March 12, 2010

Banner & Witcoff, Ltd.
Customer No. 22907

PTO/SB/26 (12-07)
Approved for use through 12/31/2007. OMB 0651-0031
U.S. Patent and Trademark Office; U.S. DEPARTMENT OF COMMERCE
Under the Paperwork Reduction Act of 1995, no persons are required to respond to a collection of information unless it displays a valid OMB control number.

TERMINAL DISCLAIMER TO OBVIATE A DOUBLE PATENTING REJECTION OVER A "PRIOR" PATENT

Docket Number (Optional): 001107.00638

In re Application of: VOGELSTEIN ET AL.

Application No.: 11709742

Filed: 23 February 2007

For: DIGITAL AMPLIFICATION

The owner*, The Johns Hopkins University, of 100 percent interest in the instant application hereby disclaims, except as provided below, the terminal part of the statutory term of any patent granted on the instant application which would extend beyond the expiration date of the full statutory term **prior patent** No. U.S. 6,440,706 as the term of said prior patent is defined in 35 U.S.C. 154 and 173, and as the term of said **prior patent** is presently shortened by any terminal disclaimer. The owner hereby agrees that any patent so granted on the instant application shall be enforceable only for and during such period that it and the **prior patent** are commonly owned. This agreement runs with any patent granted on the instant application and is binding upon the grantee, its successors or assigns.

In making the above disclaimer, the owner does not disclaim the terminal part of the term of any patent granted on the instant application that would extend to the expiration date of the full statutory term as defined in 35 U.S.C. 154 and 173 of the **prior patent**, "as the term of said **prior patent** is presently shortened by any terminal disclaimer," in the event that said **prior patent** later:

expires for failure to pay a maintenance fee;
is held unenforceable;
is found invalid by a court of competent jurisdiction;
is statutorily disclaimed in whole or terminally disclaimed under 37 CFR 1.321;
has all claims canceled by a reexamination certificate;
is reissued; or
is in any manner terminated prior to the expiration of its full statutory term as presently shortened by any terminal disclaimer.

Check either box 1 or 2 below, if appropriate.

1. ☐ For submissions on behalf of a business/organization (e.g., corporation, partnership, university, government agency, etc.), the undersigned is empowered to act on behalf of the business/organization.

I hereby declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code and that such willful false statements may jeopardize the validity of the application or any patent issued thereon.

2. ☑ The undersigned is an attorney or agent of record. Reg. No. 32,141

/Sarah A. Kagan/ — Signature

12 March 2010 — Date

Sarah A. Kagan — Typed or printed name

202 824 3000 — Telephone Number

☑ Terminal disclaimer fee under 37 CFR 1.20(d) included.

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.

*Statement under 37 CFR 3.73(b) is required if terminal disclaimer is signed by the assignee (owner).
Form PTO/SB/96 may be used for making this certification. See MPEP § 324.

Privacy Act Statement

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

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

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

Electronic Patent Application Fee Transmittal

Application Number:	11709742
Filing Date:	23-Feb-2007
Title of Invention:	Digital amplification
First Named Inventor/Applicant Name:	Bert Vogelstein
Filer:	Sarah Anne Kagan.
Attorney Docket Number:	001107.00638

Filed as Large Entity

Utility under 35 USC 111(a) Filing Fees

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

Description	Fee Code	Quantity	Amount	Sub-Total in USD($)
Miscellaneous:				
Statutory disclaimer	1814	1	140	140
		Total in USD ($)		**140**

Electronic Acknowledgement Receipt

EFS ID:	7201041
Application Number:	11709742
International Application Number:	
Confirmation Number:	3875
Title of Invention:	Digital amplification
First Named Inventor/Applicant Name:	Bert Vogelstein
Customer Number:	22907
Filer:	Sarah Anne Kagan.
Filer Authorized By:	
Attorney Docket Number:	001107.00638
Receipt Date:	12-MAR-2010
Filing Date:	23-FEB-2007
Time Stamp:	15:56:16
Application Type:	Utility under 35 USC 111(a)

Payment information:

Submitted with Payment	yes
Payment Type	Deposit Account
Payment was successfully received in RAM	$140
RAM confirmation Number	2439
Deposit Account	190733
Authorized User	

File Listing:

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

1	Oath or Declaration filed	oridec00638.pdf	122935 56a216139c5708ddb0208be63e2fc3fbb596518c	no	2
Warnings:					
Information:					
2	Oath or Declaration filed	recognition00638.pdf	68309 a750f6514789373c85619587f858118e2c381f19	no	2
Warnings:					
Information:					
3	Amendment/Req. Reconsideration-After Non-Final Reject	response00638.pdf	67041 68781bbb8ff7c932233abc71dd8c83e4a843de4b	no	1
Warnings:					
Information:					
4	Terminal Disclaimer Filed	TD00638.pdf	176717 b8d453c5dbb9874d7b33c07adbf342f88ea95d23	no	2
Warnings:					
Information:					
5	Fee Worksheet (PTO-875)	fee-info.pdf	29593 9cc8f5803a9b7daabbfdcdb820663de04a9359b7	no	2
Warnings:					
Information:					
		Total Files Size (in bytes):	464595		

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.

JOINT DECLARATION FOR PATENT APPLICATION

As the below named inventor, we hereby declare that:

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

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

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

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

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

Prior Foreign Application(s)

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

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

Prior United States Provisional Application(s)

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

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

Prior United States Application(s)

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

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

Power of Attorney

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

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

All correspondence and telephone communications should be addressed to:

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

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

We hereby declare that all statements made herein of our own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code and that such willful false statements may jeopardize the validity of the application or any patent issuing thereon.

Signature Bert Vogelstein Date 11/28/00
Full Name of First Inventor: Family Name: Vogelstein; First Given Name: Bert; Second Given Name:
Residence: Baltimore, Maryland; Citizenship: United States
Post Office Address: 3700 Breton Way, Baltimore, Maryland 21208

Signature Kenneth W. Kinzler Date 11/28/00
Full Name of Second Inventor: Family Name: Kinzler; First Given Name: Kenneth; Second Given Name: W.
Residence: BelAir, Maryland; Citizenship: United States
Post Office Address: 1403 Halkirk Way, BelAir, Maryland 21015

PATENT

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re Application of	)	Group Art Unit: 1637
	)	
Bert VOGELSTEIN et al	)	Examiner: Samuel Woolwine
	)	
Serial No. 11/709,742	)	Confirmation No. 3875
	)	
Filed: February 23, 2007	)	Atty. Dkt. No. 001107.00638
	)	
For: DIGITAL AMPLIFICATION	)	

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

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

Sir:

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

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

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

Respectfully submitted,
BANNER & WITCOFF, LTD.

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

Date: March 12, 2010

Banner & Witcoff, Ltd.
Customer No. 22907

PTO/SB/08a (01-10)
Approved for use through 07/31/2012. OMB 0651-0031
U.S. Patent and Trademark Office; U.S. DEPARTMENT OF COMMERCE

INFORMATION DISCLOSURE STATEMENT BY APPLICANT

(Not for submission under 37 CFR 1.99)

Application Number	11709742
Filing Date	2007-02-23
First Named Inventor	VOGELSTEIN, Bert
Art Unit	1637
Examiner Name	WOOLWINE, Samuel C.
Attorney Docket Number	001107.00638

U.S.PATENTS Remove

Examiner Initial*	Cite No	Patent Number	Kind Code[1]	Issue Date	Name of Patentee or Applicant of cited Document	Pages,Columns,Lines where Relevant Passages or Relevant Figures Appear
	1					

If you wish to add additional U.S. Patent citation information please click the Add button. Add

U.S.PATENT APPLICATION PUBLICATIONS Remove

Examiner Initial*	Cite No	Publication Number	Kind Code[1]	Publication Date	Name of Patentee or Applicant of cited Document	Pages,Columns,Lines where Relevant Passages or Relevant Figures Appear
	1					

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FOREIGN PATENT DOCUMENTS Remove

Examiner Initial*	Cite No	Foreign Document Number[3]	Country Code[2] i	Kind Code[4]	Publication Date	Name of Patentee or Applicant of cited Document	Pages,Columns,Lines where Relevant Passages or Relevant Figures Appear	T[5]
	1							☐

If you wish to add additional Foreign Patent Document citation information please click the Add button Add

NON-PATENT LITERATURE DOCUMENTS Remove

Examiner Initials*	Cite No	Include name of the author (in CAPITAL LETTERS), title of the article (when appropriate), title of the item (book, magazine, journal, serial, symposium, catalog, etc), date, pages(s), volume-issue number(s), publisher, city and/or country where published.	T[5]

INFORMATION DISCLOSURE STATEMENT BY APPLICANT (Not for submission under 37 CFR 1.99)		
	Application Number	11709742
	Filing Date	2007-02-23
	First Named Inventor	VOGELSTEIN, Bert
	Art Unit	1637
	Examiner Name	WOOLWINE, Samuel C.
	Attorney Docket Number	001107.00638

	1	Newton, PCR Essential Data, pages 51-52, 1995	☐

If you wish to add additional non-patent literature document citation information please click the Add button Add

EXAMINER SIGNATURE

Examiner Signature		Date Considered	

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

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

INFORMATION DISCLOSURE STATEMENT BY APPLICANT (Not for submission under 37 CFR 1.99)		
	Application Number	11709742
	Filing Date	2007-02-23
	First Named Inventor	VOGELSTEIN, Bert
	Art Unit	1637
	Examiner Name	WOOLWINE, Samuel C.
	Attorney Docket Number	001107.00638

CERTIFICATION STATEMENT

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

☐ That each item of information contained in the information disclosure statement was first cited in any communication from a foreign patent office in a counterpart foreign application not more than three months prior to the filing of the information disclosure statement. See 37 CFR 1.97(e)(1).

OR

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

☐ See attached certification statement.

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

☐ None

SIGNATURE

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

Signature	/Sarah A. Kagan/	Date (YYYY-MM-DD)	2010-03-05
Name/Print	Sarah A. Kagan	Registration Number	32141

This collection of information is required by 37 CFR 1.97 and 1.98. The information is required to obtain or retain a benefit by the public which is to file (and by the USPTO to process) an application. Confidentiality is governed by 35 U.S.C. 122 and 37 CFR 1.14. This collection is estimated to take 1 hour to complete, including gathering, preparing and submitting the completed application form to the USPTO. Time will vary depending upon the individual case. Any comments on the amount of time you require to complete this form and/or suggestions for reducing this burden, should be sent to the Chief Information Officer, U.S. Patent and Trademark Office, U.S. Department of Commerce, P.O. Box 1450, Alexandria, VA 22313-1450. DO NOT SEND FEES OR COMPLETED FORMS TO THIS ADDRESS. **SEND TO: Commissioner for Patents, P.O. Box 1450, Alexandria, VA 22313-1450.**

Privacy Act Statement

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

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

1. The information on this form will be treated confidentially to the extent allowed under the Freedom of Information Act (5 U.S.C. 552) and the Privacy Act (5 U.S.C. 552a). Records from this system of records may be disclosed to the Department of Justice to determine whether the Freedom of Information Act requires disclosure of these record s.

2. A record from this system of records may be disclosed, as a routine use, in the course of presenting evidence to a court, magistrate, or administrative tribunal, including disclosures to opposing counsel in the course of settlement negotiations.

3. A record in this system of records may be disclosed, as a routine use, to a Member of Congress submitting a request involving an individual, to whom the record pertains, when the individual has requested assistance from the Member with respect to the subject matter of the record.

4. A record in this system of records may be disclosed, as a routine use, to a contractor of the Agency having need for the information in order to perform a contract. Recipients of information shall be required to comply with the requirements of the Privacy Act of 1974, as amended, pursuant to 5 U.S.C. 552a(m).

5. A record related to an International Application filed under the Patent Cooperation Treaty in this system of records may be disclosed, as a routine use, to the International Bureau of the World Intellectual Property Organization, pursuant to the Patent Cooperation Treaty.

6. A record in this system of records may be disclosed, as a routine use, to another federal agency for purposes of National Security review (35 U.S.C. 181) and for review pursuant to the Atomic Energy Act (42 U.S.C. 218(c)).

7. A record from this system of records may be disclosed, as a routine use, to the Administrator, General Services, or his/her designee, during an inspection of records conducted by GSA as part of that agency's responsibility to recommend improvements in records management practices and programs, under authority of 44 U.S.C. 2904 and 2906. Such disclosure shall be made in accordance with the GSA regulations governing inspection of records for this purpose, and any other relevant (i.e., GSA or Commerce) directive. Such disclosure shall not be used to make determinations about individuals.

8. A record from this system of records may be disclosed, as a routine use, to the public after either publication of the application pursuant to 35 U.S.C. 122(b) or issuance of a patent pursuant to 35 U.S.C. 151. Further, a record may be disclosed, subject to the limitations of 37 CFR 1.14, as a routine use, to the public if the record was filed in an application which became abandoned or in which the proceedings were terminated and which application is referenced by either a published application, an application open to public inspections or an issued patent.

9. A record from this system of records may be disclosed, as a routine use, to a Federal, State, or local law enforcement agency, if the USPTO becomes aware of a violation or potential violation of law or regulation.

Electronic Patent Application Fee Transmittal

Application Number:	11709742
Filing Date:	23-Feb-2007
Title of Invention:	Digital amplification
First Named Inventor/Applicant Name:	Bert Vogelstein
Filer:	Sarah Anne Kagan.
Attorney Docket Number:	001107.00638

Filed as Large Entity

Utility under 35 USC 111(a) Filing Fees

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

Description	Fee Code	Quantity	Amount	Sub-Total in USD($)
Miscellaneous:				
Submission- Information Disclosure Stmt	1806	1	180	180
		Total in USD ($)		**180**

Electronic Acknowledgement Receipt

EFS ID:	7150524
Application Number:	11709742
International Application Number:	
Confirmation Number:	3875
Title of Invention:	Digital amplification
First Named Inventor/Applicant Name:	Bert Vogelstein
Customer Number:	22907
Filer:	Sarah Anne Kagan.
Filer Authorized By:	
Attorney Docket Number:	001107.00638
Receipt Date:	05-MAR-2010
Filing Date:	23-FEB-2007
Time Stamp:	14:29:38
Application Type:	Utility under 35 USC 111(a)

Payment information:

Submitted with Payment	yes
Payment Type	Deposit Account
Payment was successfully received in RAM	$180
RAM confirmation Number	731
Deposit Account	190733
Authorized User	

File Listing:

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

1	NPL Documents	newtonreference.PDF	409382 fc9d7340a9417dec08ae29b1950fbd3bce7bbdb4	no	4
Warnings:					
Information:					
2	Information Disclosure Statement (IDS) Filed (SB/08)	ids.PDF	612163 ce5a6895c8c33bba72128eff04a171acdc182b3c	no	4
Warnings:					
Information:					

A U.S. Patent Number Citation or a U.S. Publication Number Citation is required in the Information Disclosure Statement (IDS) form for autoloading of data into USPTO systems. You may remove the form to add the required data in order to correct the Informational Message if you are citing U.S. References. If you chose not to include U.S. References, the image of the form will be processed and be made available within the Image File Wrapper (IFW) system. However, no data will be extracted from this form. Any additional data such as Foreign Patent Documents or Non Patent Literature will be manually reviewed and keyed into USPTO systems.

3	Fee Worksheet (PTO-875)	fee-info.pdf	29703 05aa1c5af2b582a937237eedeef4403a3d075e5d	no	2
Warnings:					
Information:					
		Total Files Size (in bytes):	1051248		

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

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

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

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

UNITED STATES PATENT AND TRADEMARK OFFICE

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

APPLICATION NO.	FILING DATE	FIRST NAMED INVENTOR	ATTORNEY DOCKET NO.	CONFIRMATION NO.
11/709,742	02/23/2007	Bert Vogelstein	001107.00638	3875

22907 7590 12/29/2009

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

EXAMINER	
WOOLWINE, SAMUEL C	
ART UNIT	**PAPER NUMBER**
1637	

MAIL DATE	DELIVERY MODE
12/29/2009	PAPER

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

The time period for reply, if any, is set in the attached communication.

Office Action Summary	Application No.	Applicant(s)	
	11/709,742	VOGELSTEIN ET AL.	
	Examiner	Art Unit	
	SAMUEL C. WOOLWINE	1637	

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

Period for Reply

A SHORTENED STATUTORY PERIOD FOR REPLY IS SET TO EXPIRE *3* MONTH(S) OR THIRTY (30) DAYS, WHICHEVER IS LONGER, FROM THE MAILING DATE OF THIS COMMUNICATION.

- Extensions of time may be available under the provisions of 37 CFR 1.136(a). In no event, however, may a reply be timely filed after SIX (6) MONTHS from the mailing date of this communication.
- If NO period for reply is specified above, the maximum statutory period will apply and will expire SIX (6) MONTHS from the mailing date of this communication.
- Failure to reply within the set or extended period for reply will, by statute, cause the application to become ABANDONED (35 U.S.C. § 133). Any reply received by the Office later than three months after the mailing date of this communication, even if timely filed, may reduce any earned patent term adjustment. See 37 CFR 1.704(b).

Status

1)☒ Responsive to communication(s) filed on *12 October 2009*.

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) *39-41,43 and 45-69* is/are pending in the application.

4a) Of the above claim(s) *45-47,55,56,64 and 68* is/are withdrawn from consideration.

5)☐ Claim(s) _____ is/are allowed.

6)☒ Claim(s) *39,48,51,52,61,62,65,66 and 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).

Replacement drawing sheet(s) including the correction is required if the drawing(s) is objected to. See 37 CFR 1.121(d).

11)☐ 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

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

a)☐ All b)☐ Some * c)☐ None of:

1.☐ Certified copies of the priority documents have been received.

2.☐ Certified copies of the priority documents have been received in Application No. _____.

3.☐ Copies of the certified copies of the priority documents have been received in this National Stage application from the International Bureau (PCT Rule 17.2(a)).

* See the attached detailed Office action for a list of the certified copies not received.

Attachment(s)

1) ☒ Notice of References Cited (PTO-892)
2) ☐ Notice of Draftsperson's Patent Drawing Review (PTO-948)
3) ☒ Information Disclosure Statement(s) (PTO/SB/08) Paper No(s)/Mail Date *See Continuation Sheet*.
4) ☐ Interview Summary (PTO-413) Paper No(s)/Mail Date. _____ .
5) ☐ Notice of Informal Patent Application
6) ☐ Other: _____.

Continuation of Attachment(s) 3). Information Disclosure Statement(s) (PTO/SB/08), Paper No(s)/Mail Date :02/23/2007;12/18/2008;04/22/2009.

DETAILED ACTION

Election/Restrictions

Applicant's election of Group I, claims 39-41, 43, 49, 50, 53, 54, 57-60, 63, 65-67 and claims 48, 51, 52, 61, 62 and 69 in part, in the reply filed on 10/12/2009 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 45-47, 55, 56, 64 and 68 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 10/12/2009.

Double Patenting

The nonstatutory double patenting rejection is based on a judicially created doctrine grounded in public policy (a policy reflected in the statute) so as to prevent the unjustified or improper timewise extension of the "right to exclude" granted by a patent and to prevent possible harassment by multiple assignees. A nonstatutory obviousness-type double patenting rejection is appropriate where the conflicting claims are not identical, but at least one examined application claim is not patentably distinct from the reference claim(s) because the examined application claim is either anticipated by, or would have been obvious over, the reference claim(s). See, e.g., *In re Berg*, 140 F.3d 1428, 46 USPQ2d 1226 (Fed. Cir. 1998); *In re Goodman*, 11 F.3d 1046, 29 USPQ2d 2010 (Fed. Cir. 1993); *In re Longi*, 759 F.2d 887, 225 USPQ 645 (Fed. Cir. 1985); *In re Van Ornum*, 686 F.2d 937, 214 USPQ 761 (CCPA 1982); *In re Vogel*, 422 F.2d 438, 164 USPQ 619 (CCPA 1970); and *In re Thorington*, 418 F.2d 528, 163 USPQ 644 (CCPA 1969).

A timely filed terminal disclaimer in compliance with 37 CFR 1.321(c) or 1.321(d) may be used to overcome an actual or provisional rejection based on a nonstatutory double patenting ground provided the conflicting application or patent either is shown to be commonly owned with this application, or claims an invention made as a result of activities undertaken within the scope of a joint research agreement.

Effective January 1, 1994, a registered attorney or agent of record may sign a terminal disclaimer. A terminal disclaimer signed by the assignee must fully comply with 37 CFR 3.73(b).

Claims 39, 48, 51, 52, 61, 62, 65, 66 and 69 are rejected on the ground of nonstatutory obviousness-type double patenting as being unpatentable over claims 1, 3, 10, 11, 24, 28, 38, 42, 43, 56, 60, and 64 of U.S. Patent No. 6,440,706. Although the conflicting claims are not identical, they are not patentably distinct from each other because the only differences between the issued claims and the instant claims are differences in scope.

For example, with regard to instant claims 39 and 65, both issued claims 1 and 38 disclose amplifying multiple assay samples derived from a biological sample, and analyzing the amplified assay samples to determine a first number of assay samples containing a selected genetic sequence and a second number of assay samples containing a reference genetic sequence. Issued claims 1 and 38 also disclose comparing the first number to the second number to "ascertain a ratio which reflects the composition of the biological sample". Issued claim 64 discloses that the selected genetic sequence and reference genetic sequence are on distinct chromosomes.

With regard to instant claims 39 and 66, issued claim 3 discloses that between 0.1 and 0.9 of the assay samples yield an amplification product.

With regard to instant claims 48 and 69, issued claims 24 and 56 disclose that the sample is from blood.

With regard to instant claims 51 and 52, issued claim 3 discloses an overlapping range. As discussed at MPEP 2144.05 (I): "In the case where the claimed ranges

“overlap or lie inside ranges disclosed by the prior art” a *prima facie* case of obviousness exists. In re Wertheim, 541 F.2d 257, 191 USPQ 90 (CCPA 1976); In re Woodruff, 919 F.2d 1575, 16 USPQ2d 1934 (Fed. Cir. 1990)".

With regard to instant claims 61 and 62, issued claims 10, 11, 42 and 43 disclose the number of assay samples is greater than 500, or greater than 1000.

The issued claims do not expressly disclose ascertaining "allelic imbalance". However, issued claims 28 and 60 disclose that the selected genetic sequence one which is "amplified during neoplastic development". It is asserted that this represents, in fact, a form of "allelic imbalance" since whatever markers have been "amplified during neoplastic development" would be out of balance with the rest of the genome.

Conclusion

Any inquiry concerning this communication or earlier communications from the examiner should be directed to SAMUEL C. 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/
Examiner, Art Unit 1637

Notice of References Cited	Application/Control No. 11/709,742	Applicant(s)/Patent Under Reexamination VOGELSTEIN ET AL.	
	Examiner SAMUEL C. WOOLWINE	Art Unit 1637	Page 1 of 1

U.S. PATENT DOCUMENTS

*		Document Number Country Code-Number-Kind Code	Date MM-YYYY	Name	Classification
	A	US-			
	B	US-			
	C	US-			
	D	US-			
	E	US-			
	F	US-			
	G	US-			
	H	US-			
	I	US-			
	J	US-			
	K	US-			
	L	US-			
	M	US-			

FOREIGN PATENT DOCUMENTS

*		Document Number Country Code-Number-Kind Code	Date MM-YYYY	Country	Name	Classification
	N					
	O					
	P					
	Q					
	R					
	S					
	T					

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*		Include as applicable: Author, Title Date, Publisher, Edition or Volume, Pertinent Pages)
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	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.

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BIB DATA SHEET

CONFIRMATION NO. 3875

SERIAL NUMBER	FILING or 371(c) DATE RULE	CLASS	GROUP ART UNIT	ATTORNEY DOCKET NO.
11/709,742	02/23/2007	435	1637	001107.00638

APPLICANTS

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

**** CONTINUING DATA ***************************

This application is a CON of 10/828,295 04/21/2004 ABN
which is a DIV of 09/981,356 10/12/2001 PAT 6,753,147
which is a CON of 09/613,826 07/11/2000 PAT 6,440,706
which claims benefit of 60/146,792 08/02/1999

**** FOREIGN APPLICATIONS ***************************

**** IF REQUIRED, FOREIGN FILING LICENSE GRANTED **** ** SMALL ENTITY **
03/26/2008

Foreign Priority claimed ☐ Yes ☑ No 35 USC 119(a-d) conditions met ☐ Yes ☐ No Verified and Acknowledged /SAMUEL C WOOLWINE/ Examiner's Signature	☐ Met after Allowance Initials	STATE OR COUNTRY	SHEETS DRAWINGS	TOTAL CLAIMS	INDEPENDENT CLAIMS
		MD	7	48	5

ADDRESS

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

TITLE

Digital amplification

FILING FEE RECEIVED		
1605	FEES: Authority has been given in Paper No._____ to charge/credit DEPOSIT ACCOUNT No._____ for following:	☐ All Fees ☐ 1.16 Fees (Filing) ☐ 1.17 Fees (Processing Ext. of time) ☐ 1.18 Fees (Issue) ☐ Other _____ ☐ Credit

EAST Search History

EAST Search History (Prior Art)

Ref #	Hits	Search Query	DBs	Default Operator	Plurals	Time Stamp
L1	2	("6753147" "6440706").pn.	USPAT	OR	OFF	2009/12/22 12:21
L2	57	(allel$2 adj1 imbalance) same (amplified amplification duplication)	US-PGPUB; USPAT; USOCR	OR	OFF	2009/12/22 13:30
L3	4	l2 and (@ad<"19990802" @pd<"19990802")	US-PGPUB; USPAT; USOCR	OR	OFF	2009/12/22 13:31
L4	184	(allel$2 adj1 imbalance)	US-PGPUB; USPAT; USOCR	OR	OFF	2009/12/22 13:33
L5	4	l3 and (@ad<"19990802" @pd<"19990802")	US-PGPUB; USPAT; USOCR	OR	OFF	2009/12/22 13:34
L6	14	(gene adj1 duplication) same (cancer neoplas$3) and (@ad<"19990802" @pd<"19990802")	US-PGPUB; USPAT; USOCR	OR	OFF	2009/12/22 13:49
L7	216	(gene adj1 amplification) same (oncogen$4) and (@ad<"19990802" @pd<"19990802")	US-PGPUB; USPAT; USOCR	OR	OFF	2009/12/22 13:50
L8	42	l7 and (reference near3 (sequence$1 marker$1 gene$1))	US-PGPUB; USPAT; USOCR	OR	OFF	2009/12/22 13:51
L9	72	(allelic adj1 imbalance) and (reference near3 (sequence $1 marker$1 gene$1))	US-PGPUB; USPAT; USOCR	OR	OFF	2009/12/22 13:52
L10	9	l9 and (@ad<"19990802" @pd<"19990802")	US-PGPUB; USPAT; USOCR	OR	OFF	2009/12/22 13:52
L11	75	(allelic adj1 imbalance) with (detect$3 assay$3 determin$5)	US-PGPUB; USPAT; USOCR	OR	OFF	2009/12/22 13:59
L12	2	l11 and (@ad<"19990802" @pd<"19990802")	US-PGPUB; USPAT; USOCR	OR	OFF	2009/12/22 13:59
L13	12	(allel$2 adj1 imbalance) same (gene adj1 (amplification duplication))	US-PGPUB; USPAT; USOCR	OR	OFF	2009/12/22 14:10

L14	2	l12 and (@ad<"19990802" @pd<"19990802")	US-PGPUB; USPAT; USOCR	OR	OFF	2009/12/22 14:10
L15	1	l13 and (@ad<"19990802" @pd<"19990802")	US-PGPUB; USPAT; USOCR	OR	OFF	2009/12/22 14:11
L16	136	(loh (loss adj2 heterozygosity)) same (gene adj1 (amplification duplication))	US-PGPUB; USPAT; USOCR	OR	OFF	2009/12/22 14:13
L17	27	l16 and (@ad<"19990802" @pd<"19990802")	US-PGPUB; USPAT; USOCR	OR	OFF	2009/12/22 14:13

EAST Search History (Interference)

<This search history is empty>

12/22/2009 2:24:44 PM

Search Notes	Application/Control No.	Applicant(s)/Patent Under Reexamination
	11709742	VOGELSTEIN ET AL.
	Examiner	Art Unit
	SAMUEL C WOOLWINE	1637

SEARCHED			
Class	Subclass	Date	Examiner

SEARCH NOTES		
Search Notes	Date	Examiner
Prosecution history of parent applications, keyword search in EAST (see printouts)	12/22/2009	SCW

INTERFERENCE SEARCH			
Class	Subclass	Date	Examiner

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INFORMATION DISCLOSURE STATEMENT BY APPLICANT

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Sheet 1 of 3

Complete if Known	
Application Number	TBA 3
Filing Date	February 22, 2007
First Named Inventor	Bert Vogelstein et al.
Prior Group Art Unit	1637
Prior Examiner Name	M. Baughman
Attorney Docket Number	001107.00638

U.S. PATENT DOCUMENTS

Examiner Initials *	Cite No.[1]	Document Number Number - Kind Code[2] (*if known*)	Publication Date MM-DD-YYYY	Name of Patentee or Applicant of Cited Document	Pages, Columns, Lines, Where Relevant Passages or Relevant Figures Appear
		US-5,213,961	05-25-93	Bunn et al	
		US-5,736,333	04-07-98	Livak et al	
		US-5,518,901	05-21-96	Murtagh	
		US-5,804,383	09-08-1998	Gruenert et al.	
		US- 5,858,663	01-12-1999	Nisson et al.	
		US- 5,670,325	09-1997	Lapidus et al. *	
		US- 6,037,130	03-14-2000	Tyagi et al.	
		US- 5,925,517	07-20-1999	Tyagi et al.	
		US- 5,928,870	07-1999	Lapidus et al. *	
		US- 6,020,137	02-2000	Lapidus et al. *	
		US- 6,143,496	11-2000	Brown et al. *	
		US- 6,291,163	09-18-01	Sidransky	
		US-			
		US-			
		US-			
		US-			
		US-			
		US-			

FOREIGN PATENT DOCUMENTS

Examiner Initials*	Cite No.[1]	Foreign Patent Document Country Code[3] - Number[4] - Kind Code[5] (*if known*)	Publication Date MM-DD-YYYY	Name of Patentee or Applicant of Cited Document	Pages, Columns, Lines, Where Relevant Passages or Relevant Figures Appear	T[6]
		WO 95/13399	05-18-1995			
		EP 0643140 A	03-15-1995			
		WO 99/13113	03-18-1999			

Examiner Signature		Date Considered	

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INFORMATION DISCLOSURE STATEMENT BY APPLICANT

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Sheet | 2 | | 3

Complete if Known	
Application Number	TBA
Filing Date	February 22, 2007
First Named Inventor	Bert Vogelstein et al.
Group Art Unit	1637
Examiner Name	M. Baughman
Attorney Docket Number	001107.00638

OTHER PRIOR ART -- NON PATENT LITERATURE DOCUMENTS

Examiner Initials *	Cite No.[1]	Include name of the author (in CAPITAL LETTERS), title of the article (when appropriate), title of the item (book, magazine, journal, serial, symposium, catalog, etc.), date, page(s), volume-issue number(s), publisher, city and/or country where published.	T[2]
		A. PIATEK et al., "Molecular Beacon Sequence Analysis for Detecting Drug Resistance in Mycobacterium Tuberculosis", Nature Biotechnology, April 1998, pp. 359-363, Vol. 16, 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., "Multilex 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	
		B. VOGELSTEIN et al., "Digital PCR", Proceedings of the National Academy of Sciences of the United States, August 3, 1999, pp. 9236-9241, Vol. 96, No. 16	
		K. D.E. EVERETT et al, "Identification of nine species of the Chlamydiaceae Uisng PCR-RFLP", April 1999, pp. 803-813, Vol. 49, No. 2 *	
		Darren G. MONCKTON, *et al.*, "Minisatellite "Isoallele" Discrimination in Pseudohomozygotes by Single Molecule PCR and Variant Repeat Mapping", Genomics 11, pp. 465-467, 1991 *	
		Gualberto RUANO, *et al.*, "Haplotype of Multiple Polymorphisms Resolved by Enzymatic Amplification of Single DNA Molecules", Proc. National Science USA, 1990 * vol 87, pages 6296-6300	
		W. NAVIDI, *et al.*, "Using PCR in Preimplantation Genetic Disease Diagnosis", Human Reproduction, Vol. 6, No. 6, pp. 836-849, 1991 *	
		Hongua LI, *et al.*, "Amplification and Analysis of DNA Sequences in Single Human Sperm and Diploid Cells", Nature, Vol. 335, September 29, 1988 * pages 414-417	

Examiner Signature		Date Considered	

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Sheet 3 | 3

Complete if Known	
Application Number	TBA
Filing Date	February 22, 2007
First Named Inventor	Bert Vogelstein et al.
Group Art Unit	1637
Examiner Name	M. Baughman
Attorney Docket Number	001107.00638

OTHER PRIOR ART -- NON PATENT LITERATURE DOCUMENTS

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		Lin ZHANG, *et al.*, "Whole Genome Amplification from a Single Cell: Implications for Genetic Analysis", Proc. National Science USA, Vol. 89, pp. 5847-5851, July 1992 *	
		David SIDRANSKY, *et al.*, "Clonal Expansion of p53 Mutant Cells is Associated with Brain Tumour Progression", Nature, February 27, 1992 * vol 355, pages 846-847	
		Alec J. JEFFREYS, *et al.*, "Mutation Processes at Human Minisatellites", Electophoresis, 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", Forensic Science International, Vol. 66, pp. 129-141, 1994 *	
		Paul M. LIZARDI, *et al.*, "Mutation Detection and Single-Molecule Counting Using Isothermal Rolling-Circle Amplification", Nature Genetics, Vol. 19, July 1998 * pages 225-232	
		R. PARSONS, *et al.*, "Mismatch Repair Deficiency in Phenotypically Normal Human Cells", Science, Vol. 268, May 5 1995 * pages 738-740	
		MARRAS et al., "Multiplex Detection of Single-Nucleotide Variations Using Molecular Beacons," Genetic Analysis: Biomolecular Engineering, Feb. 1999, 14; 151-156	
		WHITCOMB et al., "Detection of PCR Products Using Self-Probing Amplicons and Fluorescence," Nature Biotechnology, August 1999, Vol. 17, 804-807	

Examiner Signature	/Samuel Woolwine/	Date Considered	12/20/2009

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INFORMATION DISCLOSURE STATEMENT BY APPLICANT

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Sheet | 1 | | 1

Complete if Known	
Application Number	11/709,742
Filing Date	February 23, 2007
First Named Inventor	Bert Vogelstein et al.
Group Art Unit	1637
Examiner Name	TBD
Attorney Docket Number	001107.00638

OTHER PRIOR ART -- NON PATENT LITERATURE DOCUMENTS

Examiner Initials *	Cite No.[1]	Include name of the author (in CAPITAL LETTERS), title of the article (when appropriate), title of the item (book, magazine, journal, serial, symposium, catalog, etc.), date, page(s), volume-issue number(s), publisher, city and/or country where published.	T[2]
/S.W./		M.J. BRISCO ET AL., "Detection and Quantitation of Neoplastic Cells in Acute Lymphoblastic Leukaemia, by Use of the Polymerase Chain Reaction," British Journal of Haematology, 1991, 79, 211-217	
/S.W./		M. J. BRISCO ET AL., "Outcome Prediction in Childhood Acute Lymphoblastic Leukaemia by Molecular Quantification of Residual Disease at the End of Induction," The Lancet, January 22, 1994, Vol. 343, pp. 196-200	

Examiner Signature	/Samuel Woolwine/	Date Considered	12/20/2009

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INFORMATION DISCLOSURE STATEMENT BY APPLICANT

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Sheet 1 | 1

Complete if Known	
Application Number	11/709,742
Filing Date	February 23, 2007
First Named Inventor	Bert Vogelstein et al.
Group Art Unit	1637
Examiner Name	TBD
Attorney Docket Number	001107.00638

OTHER PRIOR ART -- NON PATENT LITERATURE DOCUMENTS

Examiner Initials *	Cite No.[1]	Include name of the author (in CAPITAL LETTERS), title of the article (when appropriate), title of the item (book, magazine, journal, serial, symposium, catalog, etc.), date, page(s), volume-issue number(s), publisher, city and/or country where published.	T[2]
/S.W./		**P. J. SYKES, "Quantitation of Targets for PCR by Use of Limiting Dilution," BioTechniques, (1992), Vol. 13, No. 3, pp. 444-449**	

Examiner Signature	/Samuel Woolwine/	Date Considered	12/20/2009

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

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PATENT

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re Application of	)	Group Art Unit: 1637
	)	
Bert VOGELSTEIN et al	)	Examiner: Samuel Woolwine
	)	
Serial No. 11/709,742	)	Confirmation No. 3875
	)	
Filed: February 23, 2007	)	Atty. Dkt. No. 001107.00638
	)	
For: DIGITAL AMPLIFICATION	)	

RESPONSE TO RESTRICTION REQUIREMENT

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

Sir:

In response to the Office Action mailed September 18, 2009, applicants elect claim group I for examination in this application. Claim group I includes claims 39-41, 43, 49, 50, 53, 54, 57-60, 63, and 65-67, and claims 48, 51, 52, 61, 62, and 69 in-part.

No fee is believed due in connection with this response. However, should the Patent and Trademark Office determine that a fee is required, please charge our Deposit Account No. 19-0733.

Respectfully submitted,

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

Date: October 12, 2009

Banner & Witcoff, Ltd.
Customer No. 22907

Electronic Acknowledgement Receipt

EFS ID:	6243440
Application Number:	11709742
International Application Number:	
Confirmation Number:	3875
Title of Invention:	Digital amplification
First Named Inventor/Applicant Name:	Bert Vogelstein
Customer Number:	22907
Filer:	Sarah Anne Kagan./konnae berces
Filer Authorized By:	Sarah Anne Kagan.
Attorney Docket Number:	001107.00638
Receipt Date:	12-OCT-2009
Filing Date:	23-FEB-2007
Time Stamp:	14:10:35
Application Type:	Utility under 35 USC 111(a)

Payment information:

Submitted with Payment	no

File Listing:

Document Number	Document Description	File Name	File Size(Bytes)/ Message Digest	Multi Part /.zip	Pages (if appl.)
1	Response to Election / Restriction Filed	RespRE.pdf	66532 b85aa1064c53485984904e42ca39ea24182760ec	no	1

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

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

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

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APPLICATION NO.	FILING DATE	FIRST NAMED INVENTOR	ATTORNEY DOCKET NO.	CONFIRMATION NO.
11/709,742	02/23/2007	Bert Vogelstein	001107.00638	3875

22907 7590 09/18/2009

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EXAMINER	
WOOLWINE, SAMUEL C	
ART UNIT	**PAPER NUMBER**
1637	
MAIL DATE	**DELIVERY MODE**
09/18/2009	PAPER

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

The time period for reply, if any, is set in the attached communication.

Office Action Summary	Application No.	Applicant(s)	
	11/709,742	VOGELSTEIN ET AL.	
	Examiner	Art Unit	
	SAMUEL WOOLWINE	1637	

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

Period for Reply

A SHORTENED STATUTORY PERIOD FOR REPLY IS SET TO EXPIRE *1* MONTH(S) OR THIRTY (30) DAYS, WHICHEVER IS LONGER, FROM THE MAILING DATE OF THIS COMMUNICATION.

- Extensions of time may be available under the provisions of 37 CFR 1.136(a). In no event, however, may a reply be timely filed after SIX (6) MONTHS from the mailing date of this communication.
- If NO period for reply is specified above, the maximum statutory period will apply and will expire SIX (6) MONTHS from the mailing date of this communication.
- Failure to reply within the set or extended period for reply will, by statute, cause the application to become ABANDONED (35 U.S.C. § 133). Any reply received by the Office later than three months after the mailing date of this communication, even if timely filed, may reduce any earned patent term adjustment. See 37 CFR 1.704(b).

Status

1)☒ Responsive to communication(s) filed on *30 June 2009*.

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) *39-41,43 and 45-69* 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) _____ is/are rejected.

7)☒ Claim(s) *39-41,43 and 45-69* 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).

Replacement drawing sheet(s) including the correction is required if the drawing(s) is objected to. See 37 CFR 1.121(d).

11)☐ 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

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

a)☐ All b)☐ Some * c)☐ None of:

1.☐ Certified copies of the priority documents have been received.

2.☐ Certified copies of the priority documents have been received in Application No. _____.

3.☐ Copies of the certified copies of the priority documents have been received in this National Stage application from the International Bureau (PCT Rule 17.2(a)).

* See the attached detailed Office action for a list of the certified copies not received.

Attachment(s)

1) ☒ Notice of References Cited (PTO-892)
2) ☐ Notice of Draftsperson's Patent Drawing Review (PTO-948)
3) ☐ Information Disclosure Statement(s) (PTO/SB/08) Paper No(s)/Mail Date _____.
4) ☐ Interview Summary (PTO-413) Paper No(s)/Mail Date. _____ .
5) ☐ Notice of Informal Patent Application
6) ☐ Other: _____.

DETAILED ACTION

Election/Restrictions

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

I. Claims 39-41, 43, 49, 50, 53, 54, 57-60, 63, 65-67, and claims 48, 51, 52, 61, 62, and 69 in-part, drawn to analysis of a selected genetic sequence on a first chromosome, and a reference genetic sequence on a second chromosome, classified in class 435, subclass 6.

II. Claims 45-47, 55, 56, 64, 68, and claims 48, 51, 52, 61, 62, and 69 in-part, drawn to analysis of a first allelic form of a marker and a second allelic form of a marker, classified in class 435, subclass 6.

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

Inventions I and II are directed to related processes (in that there are steps common to both). The related inventions are distinct if: (1) the inventions as claimed are either not capable of use together or can have a materially different design, mode of operation, function, or effect; (2) the inventions do not overlap in scope, i.e., are mutually exclusive; and (3) the inventions as claimed are not obvious variants. See MPEP § 806.05(j). In the instant case, the inventions as claimed are not capable of use together. The methods of I clearly require that the "selected genetic sequence" is on a first chromosome and the "reference genetic sequence" is on a second chromosome, whereas the methods of II require analyzing a first allelic form of a marker and a second allelic form of a marker, which by definition must be on the same chromosome. For example, the allelic forms of the IL-1B -511 SNP are on chromosome 2, because the IL-

1B gene itself is on chromosome 2 (see figure 1 and last paragraph, page 1519 of Loughlin et al, Arthritis & Rheumatism 46(6):1519-1527, June 2002). Furthermore, the inventions as claimed do not encompass overlapping subject matter, since the methods of I require the "selected genetic sequence" and the "reference genetic sequence" to be on different chromosomes, while the methods of II would require the analysis of first and second allelic forms of a marker, which cannot be on different chromosomes. In addition, Applicant, in citing support for comparing genetic sequences on "distinct chromosomes" in the preliminary amendment of 02/14/2008, referred to Table 1, last line. Table 1, last line of parent patent US 6,440,706 refers to "non-polymorphic markers". If a marker has allelic forms, as in the methods of II, the marker cannot, by definition, be "non-polymorphic". Finally, there is nothing of record to show them to be obvious variants. Hence I and II are patentably distinct processes.

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 examination burden if restriction were not required because one or more of the following reasons apply:

(a) the inventions have acquired a separate status in the art in view of their different classification;

(b) the inventions have acquired a separate status in the art due to their recognized divergent subject matter;

(c) the inventions require a different field of search (for example, searching different classes/subclasses or electronic resources, or employing different search queries);

(d) the prior art applicable to one invention would not likely be applicable to another invention;

(e) the inventions are likely to raise different non-prior art issues under 35 U.S.C. 101 and/or 35 U.S.C. 112, first paragraph.

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 on the elected invention.

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.

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/
Examiner, Art Unit 1637

Notice of References Cited	Application/Control No. 11/709,742	Applicant(s)/Patent Under Reexamination VOGELSTEIN ET AL.	
	Examiner SAMUEL WOOLWINE	Art Unit 1637	Page 1 of 1

U.S. PATENT DOCUMENTS

*		Document Number Country Code-Number-Kind Code	Date MM-YYYY	Name	Classification
	A	US-			
	B	US-			
	C	US-			
	D	US-			
	E	US-			
	F	US-			
	G	US-			
	H	US-			
	I	US-			
	J	US-			
	K	US-			
	L	US-			
	M	US-			

FOREIGN PATENT DOCUMENTS

*		Document Number Country Code-Number-Kind Code	Date MM-YYYY	Country	Name	Classification
	N					
	O					
	P					
	Q					
	R					
	S					
	T					

NON-PATENT DOCUMENTS

*		Include as applicable: Author, Title Date, Publisher, Edition or Volume, Pertinent Pages)
	U	Loughlin et al. Association of the interleukin-1 gene cluster on chromosome 2q13 with knee osteoarthritis. Arthritis & Rheumatism 46(6):1519-1527, June 2002.
	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	)	Group Art Unit: 1637
	)	
Bert VOGELSTEIN et al	)	Examiner: Samuel Woolwine
	)	
Serial No. 11/709,742	)	Confirmation No. 3875
	)	
Filed: February 23, 2007	)	Atty. Dkt. No. 001107.00638
	)	
For: DIGITAL AMPLIFICATION	)	

ELECTION AND AMENDMENT

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

Sir:

In response to the Office Action mailed June 5, 2009, applicants elect claim group III for examination in this application. Claim group III includes claims 39-48. Applicants amend the group III claims below and add additional claims. Applicant believes that claims 39-48 would continue to constitute a single invention that does not require an initial step of diluting (as in claim group II) and is not directed to cancer detection *per se* (as in claim group I). In addition, new claims 49-64 also fall within the same claim group as claims 39-48. New claims 65-69 fall within claim group II. Claims of group I have been cancelled from this application.

Please amend the application as follows:

Amendments to the claims begin on page 2 of this paper.

Amendments to the specification begin on page 9 of this paper.

IN THE CLAIMS

Please substitute the following claim set for those currently or record:

1-28. (Cancelled)

29-38. (Canceled)

39. (Currently amended) A method for determining ~~the ratio of a selected genetic sequence in a population of genetic sequences from~~ an allelic imbalance in a ~~blood~~ 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 ~~blood~~ biological sample;

analyzing the amplified molecules in the assay samples of the set to determine a first number of assay samples which contain a selected genetic sequence on a first chromosome and a second number of assay samples which contain a reference genetic sequence on a second chromosome, 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~~ between 0.1 and 0.9 of the assay samples yield an amplification product;

comparing the first number of assay samples to the second number of assay samples to ascertain an allelic imbalance in ~~a ratio which reflects the composition of~~ the ~~blood~~ biological sample.

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

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

42. (Cancelled)

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

44. (Cancelled)

45. (Currently amended) A method for determining an allelic imbalance in ~~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~~ the 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~~ a first allelic form of a marker and a second number of assay samples which contain a ~~reference non-polymorphic~~

second allelic form of the marker, wherein between 0.1 and 0.9 of the assay samples yield an amplification product ~~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 an allelic imbalance in ~~a ratio which reflects the composition of~~ the biological sample; and

identifying an allelic imbalance in the biological sample ~~based on the ratio ascertained~~.

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

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

48. (Currently amended) The method of claim 39 or 45 wherein the biological sample is from blood.

49. (New) The method of claim 39 wherein the selected genetic sequence is a non-polymorphic marker.

50. (New) The method of claim 39 wherein the reference genetic sequence is a non-polymorphic marker.

51. (New) The method of claim 39 or 45 wherein between 0.1 and 0.6 of the assay samples yield an amplification product.

52. (New) The method of claim 39 or 45 wherein between 0.3 and 0.5 of the assay samples yield an amplification product.

53. (New) The method of claim 39 wherein between 0.1 and 0.9 of the assay samples yield an amplification product as determined by amplification of the selected genetic sequence.

54. (New) The method of claim 39 wherein between 0.1 and 0.9 of the assay samples yield an amplification product as determined by amplification of the reference genetic sequence.

55. (New) The method of claim 45 wherein between 0.1 and 0.9 of the assay samples yield an amplification product as determined by amplification of the first allelic form of the marker.

56. (New) The method of claim 45 wherein between 0.1 and 0.9 of the assay samples yield an amplification product as determined by amplification of the second allelic form of the marker.

57. (New) The method of claim 39 wherein between 0.1 and 0.6 of the assay samples yield an amplification product as determined by amplification of the selected genetic sequence.

58. (New) The method of claim 39 wherein between 0.1 and 0.6 of the assay samples yield an amplification product as determined by amplification of the reference genetic sequence.

59. (New) The method of claim 39 wherein between 0.3 and 0.5 of the assay samples yield an amplification product as determined by amplification of the selected genetic sequence.

60. (New) The method of claim 39 wherein between 0.3 and 0.5 of the assay samples yield an amplification product as determined by amplification of the reference genetic sequence.

61. (New) The method of claim 39 or 45 wherein the set comprises at least 500 assay samples.

62. (New) The method of claim 39 or 45 wherein the set comprises at least 1000 assay samples.

63. (New) The method of claim 39 wherein the amplified molecules in each of the assay samples in the first and second numbers of assay samples are homogeneous such that the first number of assay samples do not contain the reference genetic sequence and the second number of assay samples do not contain the selected genetic sequence.

64. (New) The method of claim 45 wherein the amplified molecules in each of the assay samples within the first and second numbers of assay samples are homogeneous such that the first number of assay samples do not contain the second allelic form of the marker and the second number of assay samples do not contain the first allelic form of the marker.

65. (New) A method for determining an allelic imbalance in a biological sample, comprising the steps of:

distributing nucleic acid template molecules from 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 a selected genetic sequence on a first chromosome and a second number of assay samples which contain a reference genetic sequence on a second chromosome;

comparing the first number of assay samples to the second number of assay samples to ascertain an allelic imbalance between the first chromosome and the second chromosome in the biological sample.

66. (New) The method of claim 65 wherein between 0.1 and 0.9 of the assay samples yield an amplification product.

67. (New) The method of claim 66 wherein between 0.1 and 0.9 of the assay samples yield a homogeneous amplification product.

68. (New) A method for determining an allelic imbalance in a biological sample, comprising the steps of:

distributing nucleic acid template molecules from 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 a first allelic form of a marker and a second number of assay samples which contain a second allelic form of the marker;

comparing the first number of assay samples to the second number of assay samples to ascertain an allelic imbalance between the first allelic form and the second allelic form in the biological sample.

69. (New) The method of claim 65 or 68 wherein the biological sample is blood.

IN THE SPECIFICATION

Please substitute the following paragraphs at the indicated locations:

At page 7, paragraph 1:

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~~ amplifiable template molecules. Desirably each assay sample prior to amplification will contain less than a hundred or less than ten template molecules.

At the paragraph spanning pages 16 and 17:

The second step in Fig 1A involves the detection of these PCR products. It was necessary to considerably modify the standard MB probe approach in order for it to function efficiently in Digital Amplification applications. Theoretically, one separate MB probe could be used to detect each specific mutation that might occur within the queried sequence. By inclusion of one MB corresponding to WT sequence and another corresponding to mutant sequence, the nature of the PCR product would be revealed. Though this strategy could obviously be used effectively in some situations, it becomes complex when several different

mutations are expected to occur within the same queried sequence. For example, in the *c-Ki-Ras* gene example explored here, twelve different base substitutions resulting in missense mutations could theoretically occur within codons 12 and 13, and at least seven of these are observed in naturally-occurring human cancers. To detect all twelve mutations as well as the WT sequence with individual Molecular Beacons would require 13 different probes. Inclusion of such a large number of MB probes would not only raise the background fluorescence but would be expensive. We therefore attempted to develop a single probe that would react with WT sequences better than any mutant sequence within the queried sequence. We found that the length of the loop sequence, its melting temperature, and the length and sequence of the stem were each important in determining the efficacy of such probes. Loops ranging from 14 to 26 bases and stems ranging from 4 to 6 bases, as well as numerous sequence variations of both stems and loops, were tested during the optimization procedure. For discrimination between WT and mutant sequences (MB-GREEN probe), we found that a 16 base pair loop, of melting temperature (Tm) 50-51═ °, and a 4 bp stem, of sequence 5'-CACG-3', were optimal. For MB-RED probes, the same stem, with a 19-20 bp loop of Tm 54-56═ °, proved optimal. The differences in the loop sizes and melting temperatures between MB-GREEN and MB-RED probes reflected the fact that only the GREEN probe is designed to discriminate between closely related sequences, with a shorter region of homology facilitating such discrimination.

At page 19, paragraph 1

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 ~~Amplificationassay~~ 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).

At the paragraph spanning pages 19 and 20:

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 ~~Amplificationexperiment~~ 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).

Remarks

Applicants make the amendment to the claims in order to describe the invention more distinctly. As shown below, each amendment and claim is supported by the application as originally filed, and therefore does not add prohibited new matter to the application.

Amendments to the specification and to claim 43 merely correct obvious typographical errors.

Claim No.	Claim Recitation	Specification Support	Specification Citation
39, 45	an allelic imbalance	Allelic imbalances often result from a disease state. These can be detected using digital amplification.	Sentence spanning pages 10-11; See also Table 1, last line
39	biological sample	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.	Page 11, lines 3-6
39	a selected genetic sequence on a first chromosome and a second number of assay samples which contain a reference genetic sequence on a	Probe 1 detects marker sequence; Probe 2 detects marker sequence from another chromosome	Table 1

	second chromosome		
39, 45	between 0.1 and 0.9 of the assay samples yield an amplification product;	To achieve a dilution to approximately a single template molecule level, one can dilute such that between 0.1 and 0.9 of the assay samples yield an amplification product.	Page 9, lines 26-28
45	a first allelic form of a marker	Allelic discrimination	Table 1, application # 6.
49	selected genetic sequence is a non-polymorphic marker.	Quantitative analysis with non-polymorphic markers	Table 1, example # 7.
50	reference genetic sequence is a non-polymorphic marker.	Quantitative analysis with non-polymorphic markers	Table 1, example # 7.
51, 57-58	between 0.1 and 0.6 of the assay samples yield an amplification product.	More preferably the dilution will be to between 0.1 and 0.6	Page 9, line 28 to page 10, line 1
52, 59-60	0.3 and 0.5 of the assay samples yield an amplification product.	more preferably to between 0.3 and 0.5 of the assay samples yielding an amplification product.	Page 10, line 1
53	between 0.1 and 0.9 of the assay samples yield an amplification product as determined by the selected genetic sequence.	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	Page 9, lines 16-28

		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.	
54	between 0.1 and 0.9 of the assay samples yield an amplification product as determined by the reference genetic sequence.	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	Page 9, lines 16-28

		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.	
55	wherein between 0.1 and 0.9 of the assay samples yield an amplification product as determined by the first allelic form of the marker.	Allelic discrimination; 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	Table 1, application # 6; Page 9, lines 16-28.

		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.	
56	between 0.1 and 0.9 of the assay samples yield an amplification product as determined by the second allelic	Allelic discrimination; In one preferred embodiment each diluted sample has on average one half a	Table 1, application # 6; Page 9, lines 16-28.

	form of the marker.	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.	
61	at least 500 assay samples.	More preferably at least 15, 20, 25, 30,	Page 10, lines 5-6

		40, 50, 75, 100, 500, or 1000 diluted assay samples are amplified and analyzed.	
62	at least 1000 assay samples.	More preferably at least 15, 20, 25, 30, 40, 50, 75, 100, 500, or 1000 diluted assay samples are amplified and analyzed.	Page 10, lines 5-6
63	wherein the amplified molecules in each of the assay samples in the first and second numbers of assay samples are homogeneous such that the first number of assay samples do not contain the reference genetic sequence and the second number of assay samples do not contain the selected genetic sequence.	If the analysis method being used requires 100% analyte to detect, then dilution down to the single template molecule level will be required. 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. The presence of homogeneous WT or mutant sequence confirmed that the amplification products were usually derived from single template molecules.	Page 9, lines 23-25; Page 15, lines 1-3; Page 19, lines 20-22.
64	wherein the amplified molecules in each of the assay samples within the first and second numbers of	If the analysis method being used requires 100% analyte to detect, then dilution down to the single	Page 9, lines 23-25; Page 15, lines 1-3; Page 19, lines 20-22.

	assay samples are homogeneous such that the first number of assay samples do not contain the second allelic form of the marker and the second number of assay samples do not contain the first allelic form of the marker.	template molecule level will be required. 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. The presence of homogeneous WT or mutant sequence confirmed that the amplification products were usually derived from single template molecules.	
65, 68	distributing nucleic acid template molecules from a biological sample to form a set comprising a plurality of assay samples;	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. The digital amplification method requires analysis of a large number of samples to get meaningful results.	Page 6, lines 17-20; Page 10, lines 3-4; Page 7, lines 13-15

		The dilution can be performed from more concentrated samples. Alternatively, dilute sources of template nucleic acids can be used.	
67	between 0.1 and 0.9 of the assay samples yield a homogeneous amplification product.	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.	Page 9, lines 23-28.

No excess claim fees are believed to be due, because fewer independent and fewer total claims are presented here than were previously paid for. However, if fees are due, please charge any necessary fees to our deposit account no. 19-0733.

Respectfully submitted,

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

Date: June 30, 2009

Banner & Witcoff, Ltd.
Customer No. 22907

Electronic Acknowledgement Receipt

EFS ID:	5613787
Application Number:	11709742
International Application Number:	
Confirmation Number:	3875
Title of Invention:	Digital amplification
First Named Inventor/Applicant Name:	Bert Vogelstein
Customer Number:	22907
Filer:	Sarah Anne Kagan.
Filer Authorized By:	
Attorney Docket Number:	001107.00638
Receipt Date:	30-JUN-2009
Filing Date:	23-FEB-2007
Time Stamp:	13:18:15
Application Type:	Utility under 35 USC 111(a)

Payment information:

Submitted with Payment	no

File Listing:

Document Number	Document Description	File Name	File Size(Bytes)/ Message Digest	Multi Part /.zip	Pages (if appl.)
1	Response to Election / Restriction Filed	election00638.pdf	146496 d42a4cc580e8a6477a14d8913f7d197079fd0d07	no	22

Warnings:

Information:

Total Files Size (in bytes):	146496

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

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

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

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

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

PATENT APPLICATION FEE DETERMINATION RECORD

Substitute for Form PTO-875

Application or Docket Number	Filing Date	☐ To be Mailed
11/709,742	02/23/2007	

APPLICATION AS FILED – PART I

FOR	(Column 1) NUMBER FILED	(Column 2) NUMBER EXTRA	SMALL ENTITY ☒ RATE ($)	SMALL ENTITY ☒ FEE ($)	OR	OTHER THAN SMALL ENTITY RATE ($)	OTHER THAN SMALL ENTITY FEE ($)
☐ BASIC FEE (37 CFR 1.16(a), (b), or (c))	N/A	N/A	N/A			N/A	
☐ SEARCH FEE (37 CFR 1.16(k), (i), or (m))	N/A	N/A	N/A			N/A	
☐ EXAMINATION FEE (37 CFR 1.16(o), (p), or (q))	N/A	N/A	N/A			N/A	
TOTAL CLAIMS (37 CFR 1.16(i))	minus 20 =	*	X $ =		OR	X $ =	
INDEPENDENT CLAIMS (37 CFR 1.16(h))	minus 3 =	*	X $ =			X $ =	
☐ APPLICATION SIZE FEE (37 CFR 1.16(s))	If the specification and drawings exceed 100 sheets of paper, the application size fee due is $250 ($125 for small entity) for each additional 50 sheets or fraction thereof. See 35 U.S.C. 41(a)(1)(G) and 37 CFR 1.16(s).						
☐ MULTIPLE DEPENDENT CLAIM PRESENT (37 CFR 1.16(j))							
* If the difference in column 1 is less than zero, enter "0" in column 2.			TOTAL			TOTAL	

APPLICATION AS AMENDED – PART II

AMENDMENT 06/30/2009

	(Column 1) CLAIMS REMAINING AFTER AMENDMENT		(Column 2) HIGHEST NUMBER PREVIOUSLY PAID FOR	(Column 3) PRESENT EXTRA	SMALL ENTITY RATE ($)	SMALL ENTITY ADDITIONAL FEE ($)	OR	OTHER THAN SMALL ENTITY RATE ($)	OTHER THAN SMALL ENTITY ADDITIONAL FEE ($)
Total (37 CFR 1.16(i))	* 35	Minus	** 48	= 0	X $26 =	0	OR	X $ =	
Independent (37 CFR 1.16(h))	* 4	Minus	***5	= 0	X $110 =	0	OR	X $ =	
☐ Application Size Fee (37 CFR 1.16(s))									
☒ FIRST PRESENTATION OF MULTIPLE DEPENDENT CLAIM (37 CFR 1.16(j))						195	OR		
					TOTAL ADD'L FEE	195	OR	TOTAL ADD'L FEE	

AMENDMENT

	(Column 1) CLAIMS REMAINING AFTER AMENDMENT		(Column 2) HIGHEST NUMBER PREVIOUSLY PAID FOR	(Column 3) PRESENT EXTRA	RATE ($)	ADDITIONAL FEE ($)		RATE ($)	ADDITIONAL FEE ($)
Total (37 CFR 1.16(i))	*	Minus	**	=	X $ =		OR	X $ =	
Independent (37 CFR 1.16(h))	*	Minus	***	=	X $ =		OR	X $ =	
☐ Application Size Fee (37 CFR 1.16(s))									
☐ FIRST PRESENTATION OF MULTIPLE DEPENDENT CLAIM (37 CFR 1.16(j))							OR		
					TOTAL ADD'L FEE		OR	TOTAL ADD'L FEE	

* If the entry in column 1 is less than the entry in column 2, write "0" in column 3.

** If the "Highest Number Previously Paid For" IN THIS SPACE is less than 20, enter "20".

*** If the "Highest Number Previously Paid For" IN THIS SPACE is less than 3, enter "3".

The "Highest Number Previously Paid For" (Total or Independent) is the highest number found in the appropriate box in column 1.

Legal Instrument Examiner:
/CAROLYN COFER/

Document code: WFEE

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APPLICATION NO.	FILING DATE	FIRST NAMED INVENTOR	ATTORNEY DOCKET NO.	CONFIRMATION NO.
11/709,742	02/23/2007	Bert Vogelstein	001107.00638	3875

22907 7590 06/05/2009

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

EXAMINER	
WOOLWINE, SAMUEL C	
ART UNIT	PAPER NUMBER
1637	
MAIL DATE	DELIVERY MODE
06/05/2009	PAPER

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

The time period for reply, if any, is set in the attached communication.

Office Action Summary	Application No.	Applicant(s)	
	11/709,742	VOGELSTEIN ET AL.	
	Examiner	Art Unit	
	SAMUEL WOOLWINE	1637	

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

Period for Reply

A SHORTENED STATUTORY PERIOD FOR REPLY IS SET TO EXPIRE *1* MONTH(S) OR THIRTY (30) DAYS, WHICHEVER IS LONGER, FROM THE MAILING DATE OF THIS COMMUNICATION.

- Extensions of time may be available under the provisions of 37 CFR 1.136(a). In no event, however, may a reply be timely filed after SIX (6) MONTHS from the mailing date of this communication.
- If NO period for reply is specified above, the maximum statutory period will apply and will expire SIX (6) MONTHS from the mailing date of this communication.
- Failure to reply within the set or extended period for reply will, by statute, cause the application to become ABANDONED (35 U.S.C. § 133). Any reply received by the Office later than three months after the mailing date of this communication, even if timely filed, may reduce any earned patent term adjustment. See 37 CFR 1.704(b).

Status

1)☐ Responsive to communication(s) filed on _____.

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

3)☐ 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) *1-48* 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) _____ is/are rejected.

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

8)☒ Claim(s) *1-48* 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).

Replacement drawing sheet(s) including the correction is required if the drawing(s) is objected to. See 37 CFR 1.121(d).

11)☐ 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

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

a)☐ All b)☐ Some * c)☐ None of:

1.☐ Certified copies of the priority documents have been received.

2.☐ Certified copies of the priority documents have been received in Application No. _____.

3.☐ Copies of the certified copies of the priority documents have been received in this National Stage application from the International Bureau (PCT Rule 17.2(a)).

* See the attached detailed Office action for a list of the certified copies not received.

Attachment(s)

1) ☐ Notice of References Cited (PTO-892)
2) ☐ Notice of Draftsperson's Patent Drawing Review (PTO-948)
3) ☐ Information Disclosure Statement(s) (PTO/SB/08) Paper No(s)/Mail Date _____.
4) ☐ Interview Summary (PTO-413) Paper No(s)/Mail Date. _____ .
5) ☐ Notice of Informal Patent Application
6) ☐ Other: _____.

DETAILED ACTION

Election/Restrictions

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

I. Claims 1-28, drawn to methods for detecting cancer associated mutant nucleic acids, classified in class 435, subclass 6.

II. Claims 29-38, drawn to methods for determining a ratio of a selected genetic sequence in a population of genetic sequences requiring diluting a sample to form a set of assay samples, classified in class 435, subclass 6.

III. Claims 39-48, drawn to methods for determining a ratio of a selected genetic sequence in a population of genetic sequences requiring at least one-fiftieth of the assay samples in a set of samples 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, classified in class 435, subclass 6.

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

Inventions I, 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 each require limitations not required by the other inventions as claimed, therefore having different designs.

Group I requires diluting "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". This limitation is not required in Group II or III. While Group III requires "at least one-fiftieth of the assay samples in a set of samples 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", it does not require making any dilutions as required by Group I.

Group II requires "diluting nucleic acid templates…to form a set comprising a plurality of assay samples", which is not required of Group III. Group II also requires "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" and "comparing the first number to the second number to ascertain a ratio which reflects the composition of the...sample". These limitations are not required for Group I.

Group III requires "at least one-fiftieth of the assay samples in a set of samples 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", which is not required by Group II. Group III also requires "comparing the first number to the second number to ascertain a ratio which reflects the composition of the…sample", which is not required by Group I.

Therefore, each Group requires limitations not found in the other Groups.

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 examination burden if restriction were not required because one or more of the following reasons apply:

(a) the inventions have acquired a separate status in the art in view of their different classification;

(b) the inventions have acquired a separate status in the art due to their recognized divergent subject matter;

(c) the inventions require a different field of search (for example, searching different classes/subclasses or electronic resources, or employing different search queries);

(d) the prior art applicable to one invention would not likely be applicable to another invention;

(e) the inventions are likely to raise different non-prior art issues under 35 U.S.C. 101 and/or 35 U.S.C. 112, first paragraph.

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 on the elected invention.

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.

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/
Examiner, Art Unit 1637

PATENT

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re Application of:	)	Confirmation No. 3875
	)	
	)	Group Art Unit: 1637
Bert Vogelstein et al.	)	
	)	Examiner: M. Baughman
Serial No.: 11/709,742	)	
	)	
Filed: February 22, 2007	)	Atty. Dkt. No. 001107.00638
	)	
For: DIGITAL AMPLIFICATION	)	

INFORMATION DISCLOSURE STATEMENT

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

Sir:

In accordance with 37 C.F.R. §§ 1.97 and 1.98, enclosed is PTO Form-1449 listing two non-patent documents for consideration by the Examiner during the prosecution of the subject application.

Respectfully submitted,

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

Date: April 23, 2009

Banner & Witcoff, Ltd.
Customer No. 22907

PTO/SB/08B(10-01)
Approved for use through 10/31/2002. OMB 0651-0031
U.S. Patent and Trademark Office: U.S. DEPARTMENT OF COMMERCE
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Substitute for form 1449A/PTO

INFORMATION DISCLOSURE STATEMENT BY APPLICANT

(use as many sheets as necessary)

Sheet | 1 | | 1

Complete if Known	
Application Number	11/709,742
Filing Date	February 23, 2007
First Named Inventor	Bert Vogelstein et al.
Group Art Unit	1637
Examiner Name	TBD
Attorney Docket Number	001107.00638

OTHER PRIOR ART -- NON PATENT LITERATURE DOCUMENTS

Examiner Initials *	Cite No.[1]	Include name of the author (in CAPITAL LETTERS), title of the article (when appropriate), title of the item (book, magazine, journal, serial, symposium, catalog, etc.), date, page(s), volume-issue number(s), publisher, city and/or country where published.	T[2]
		M.J. BRISCO ET AL., "Detection and Quantitation of Neoplastic Cells in Acute Lymphoblastic Leukaemia, by Use of the Polymerase Chain Reaction," British Journal of Haematology, 1991, 79, 211-217	
		M. J. BRISCO ET AL., "Outcome Prediction in Childhood Acute Lymphoblastic Leukaemia by Molecular Quantification of Residual Disease at the End of Induction," The Lancet, January 22, 1994, Vol. 343, pp. 196-200	

Examiner Signature		Date Considered	

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

[1] Unique citation designation number (optional). [2] Applicant is to place a check mark here if English language Translation is attached.

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Electronic Acknowledgement Receipt

EFS ID:	5199716
Application Number:	11709742
International Application Number:	
Confirmation Number:	3875
Title of Invention:	Digital amplification
First Named Inventor/Applicant Name:	Bert Vogelstein
Customer Number:	22907
Filer:	Sarah Anne Kagan./konnae berces
Filer Authorized By:	Sarah Anne Kagan.
Attorney Docket Number:	001107.00638
Receipt Date:	22-APR-2009
Filing Date:	23-FEB-2007
Time Stamp:	16:06:35
Application Type:	Utility under 35 USC 111(a)

Payment information:

Submitted with Payment	no

File Listing:

Document Number	Document Description	File Name	File Size(Bytes)/ Message Digest	Multi Part /.zip	Pages (if appl.)
1	Preliminary Amendment	1107Prelamdt638.pdf	59666 921abfcb4dfcff29509bb36074600e3da34a67b4	no	3

Warnings:	
Information:	

2	Information Disclosure Statement (IDS) Filed (SB/08)	1107IDS638.pdf	64257 1818c140e7eaad0b709429c89725c66110eea0ec	no	2
Warnings:					
Information:					
This is not an USPTO supplied IDS fillable form					
3	NPL Documents	1stbrisco.pdf	777046 c8d8da85bb809ec5c6760cd961685686039e1e0b	no	6
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4	NPL Documents	2ndBrisco.pdf	1570141 480474e2b6e7f05aa9487b56cf1cd565a2b53409	no	13
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New Applications Under 35 U.S.C. 111

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National Stage of an International Application under 35 U.S.C. 371

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

New International Application Filed with the USPTO as a Receiving Office

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

PATENT

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re Application of	)	Prior Group Art Unit: 1637
	)	
Bert VOGELSTEIN et al	)	Prior Examiner: M. Baughman
	)	
Serial No. 11/709,742	)	Confirmation No. 3875
	)	
Filed: February 22, 2007	)	Atty. Dkt. No. 001107.00638
	)	
For: DIGITAL AMPLIFICATION	)	

PRELIMINARY AMENDMENT

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

Sir:

Prior to the examination of the above-referenced application, please amend the application as follows:

Amendments to the Specification begin on page 2 of this paper.

Remarks begin on page 3 of this paper.

IN THE SPECIFICATION:

Applicants respectfully request that the following Table 1 be added at page 9, after line 11.

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

Remarks

Please enter this amendment prior to examination on the merits. The Table was omitted inadvertently upon filing, but is supported by the incorporation-by-reference in paragraph 1 of page 1 of the specification. See, *e.g,* last page of Serial No. 60/146,792. No new matter is added by this amendment.

Please charge any necessary fees to our deposit account no. 19-0733.

Respectfully submitted,

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

Date: April 13, 2009

Banner & Witcoff, Ltd.
Customer No. 22907

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

PATENT APPLICATION FEE DETERMINATION RECORD

Substitute for Form PTO-875

Application or Docket Number	Filing Date	☐ To be Mailed
11/709,742	02/23/2007	

APPLICATION AS FILED – PART I

FOR	(Column 1) NUMBER FILED	(Column 2) NUMBER EXTRA	SMALL ENTITY ☒ RATE ($)	SMALL ENTITY ☒ FEE ($)	OR	OTHER THAN SMALL ENTITY RATE ($)	OTHER THAN SMALL ENTITY FEE ($)
☒ BASIC FEE (37 CFR 1.16(a), (b), or (c))	N/A	N/A	N/A	150		N/A	
☐ SEARCH FEE (37 CFR 1.16(k), (i), or (m))	N/A	N/A	N/A			N/A	
☐ EXAMINATION FEE (37 CFR 1.16(o), (p), or (q))	N/A	N/A	N/A			N/A	
TOTAL CLAIMS (37 CFR 1.16(i))	minus 20 =	*	X $ =		OR	X $ =	
INDEPENDENT CLAIMS (37 CFR 1.16(h))	minus 3 =	*	X $ =			X $ =	
☐ APPLICATION SIZE FEE (37 CFR 1.16(s))	If the specification and drawings exceed 100 sheets of paper, the application size fee due is $250 ($125 for small entity) for each additional 50 sheets or fraction thereof. See 35 U.S.C. 41(a)(1)(G) and 37 CFR 1.16(s).						
☐ MULTIPLE DEPENDENT CLAIM PRESENT (37 CFR 1.16(j))							
* If the difference in column 1 is less than zero, enter "0" in column 2.			TOTAL	150		TOTAL	

APPLICATION AS AMENDED – PART II

AMENDMENT 04/22/2009

	(Column 1) CLAIMS REMAINING AFTER AMENDMENT		(Column 2) HIGHEST NUMBER PREVIOUSLY PAID FOR	(Column 3) PRESENT EXTRA	SMALL ENTITY RATE ($)	SMALL ENTITY ADDITIONAL FEE ($)	OR	OTHER THAN SMALL ENTITY RATE ($)	OTHER THAN SMALL ENTITY ADDITIONAL FEE ($)
Total (37 CFR 1.16(i))	* 48	Minus	** 48	= 0	X $26 =	0	OR	X $ =	
Independent (37 CFR 1.16(h))	* 5	Minus	***5	= 0	X $110 =	0	OR	X $ =	
☐ Application Size Fee (37 CFR 1.16(s))									
☐ FIRST PRESENTATION OF MULTIPLE DEPENDENT CLAIM (37 CFR 1.16(j))							OR		
					TOTAL ADD'L FEE	0	OR	TOTAL ADD'L FEE	

AMENDMENT

	(Column 1) CLAIMS REMAINING AFTER AMENDMENT		(Column 2) HIGHEST NUMBER PREVIOUSLY PAID FOR	(Column 3) PRESENT EXTRA	RATE ($)	ADDITIONAL FEE ($)		RATE ($)	ADDITIONAL FEE ($)
Total (37 CFR 1.16(i))	*	Minus	**	=	X $ =		OR	X $ =	
Independent (37 CFR 1.16(h))	*	Minus	***	=	X $ =		OR	X $ =	
☐ Application Size Fee (37 CFR 1.16(s))									
☐ FIRST PRESENTATION OF MULTIPLE DEPENDENT CLAIM (37 CFR 1.16(j))							OR		
					TOTAL ADD'L FEE		OR	TOTAL ADD'L FEE	

* If the entry in column 1 is less than the entry in column 2, write "0" in column 3.

** If the "Highest Number Previously Paid For" IN THIS SPACE is less than 20, enter "20".

*** If the "Highest Number Previously Paid For" IN THIS SPACE is less than 3, enter "3".

The "Highest Number Previously Paid For" (Total or Independent) is the highest number found in the appropriate box in column 1.

Legal Instrument Examiner:
/GLORIA TRAMMELL/

PATENT

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re Application of:	)	Confirmation No. 3875
	)	
	)	Prior Group Art Unit: 1637
Bert Vogelstein et al.	)	
	)	Prior Examiner: M. Baughman
Serial No.: 11/709,742	)	
	)	
Filed: February 22, 2007	)	Atty. Dkt. No. 001107.00638
	)	
For: DIGITAL AMPLIFICATION	)	

INFORMATION DISCLOSURE STATEMENT

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

Sir:

In accordance with 37 C.F.R. §§ 1.97 and 1.98, enclosed is PTO Form-1449 listing a one non-patent document for consideration by the Examiner during the prosecution of the subject application.

Respectfully submitted,

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

Date: December 18, 2008

Banner & Witcoff, Ltd.
Customer No. 22907

PTO/SB/08B(10-01)
Approved for use through 10/31/2002. OMB 0651-0031
U.S. Patent and Trademark Office: U.S. DEPARTMENT OF COMMERCE
Under the Paperwork Reduction Act of 1995, no persons are required to respond to a collection of information unless it contains a valid OMB control number

Substitute for form 1449A/PTO

INFORMATION DISCLOSURE STATEMENT BY APPLICANT

(use as many sheets as necessary)

Sheet 1 | 1

Complete if Known	
Application Number	11/709,742
Filing Date	February 23, 2007
First Named Inventor	Bert Vogelstein et al.
Group Art Unit	1637
Examiner Name	TBD
Attorney Docket Number	001107.00638

OTHER PRIOR ART -- NON PATENT LITERATURE DOCUMENTS

Examiner Initials *	Cite No.[1]	Include name of the author (in CAPITAL LETTERS), title of the article (when appropriate), title of the item (book, magazine, journal, serial, symposium, catalog, etc.), date, page(s), volume-issue number(s), publisher, city and/or country where published.	T[2]
		P. J. SYKES, "Quantitation of Targets for PCR by Use of Limiting Dilution," BioTechniques, (1992), Vol. 13, No. 3, pp. 444-449	

Examiner Signature		Date Considered	

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

[1] Unique citation designation number (optional). [2] 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.

Electronic Acknowledgement Receipt

EFS ID:	4484557
Application Number:	11709742
International Application Number:	
Confirmation Number:	3875
Title of Invention:	Digital amplification
First Named Inventor/Applicant Name:	Bert Vogelstein
Customer Number:	22907
Filer:	Sarah Anne Kagan./konnae berces
Filer Authorized By:	Sarah Anne Kagan.
Attorney Docket Number:	001107.00638
Receipt Date:	18-DEC-2008
Filing Date:	23-FEB-2007
Time Stamp:	16:01:55
Application Type:	Utility under 35 USC 111(a)

Payment information:

Submitted with Payment	no

File Listing:

Document Number	Document Description	File Name	File Size(Bytes)/ Message Digest	Multi Part /.zip	Pages (if appl.)
1	Information Disclosure Statement (IDS) Filed (SB/08)	IDS638.pdf	178151 a2dc3f081272d03536d44e99fa06bafd72391b77	no	2

Warnings:	
Information:	

This is not an USPTO supplied IDS fillable form					
2	NPL Documents	Sykes638.PDF	577978 b975e56deecd4a2a3be9a321cb1a53b2e0d2ecc4	no	6
Warnings:					
Information:					
			Total Files Size (in bytes):	756129	

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

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

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

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

UNITED STATES PATENT AND TRADEMARK OFFICE

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

APPLICATION NUMBER	FILING OR 371(C) DATE	FIRST NAMED APPLICANT	ATTY. DOCKET NO./TITLE
11/709,742	02/23/2007	Bert Vogelstein	001107.00638

CONFIRMATION NO. 3875

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

PUBLICATION NOTICE

Title: Digital amplification

Publication No.US-2008-0241830-A1
Publication Date:10/02/2008

NOTICE OF PUBLICATION OF APPLICATION

The above-identified application will be electronically published as a patent application publication pursuant to 37 CFR 1.211, et seq. The patent application publication number and publication date are set forth above.

The publication may be accessed through the USPTO's publically available Searchable Databases via the Internet at www.uspto.gov. The direct link to access the publication is currently http://www.uspto.gov/patft/.

The publication process established by the Office does not provide for mailing a copy of the publication to applicant. A copy of the publication may be obtained from the Office upon payment of the appropriate fee set forth in 37 CFR 1.19(a)(1). Orders for copies of patent application publications are handled by the USPTO's Office of Public Records. The Office of Public Records can be reached by telephone at (703) 308-9726 or (800) 972-6382, by facsimile at (703) 305-8759, by mail addressed to the United States Patent and Trademark Office, Office of Public Records, Alexandria, VA 22313-1450 or via the Internet.

In addition, information on the status of the application, including the mailing date of Office actions and the dates of receipt of correspondence filed in the Office, may also be accessed via the Internet through the Patent Electronic Business Center at www.uspto.gov using the public side of the Patent Application Information and Retrieval (PAIR) system. The direct link to access this status information is currently http://pair.uspto.gov/. Prior to publication, such status information is confidential and may only be obtained by applicant using the private side of PAIR.

Further assistance in electronically accessing the publication, or about PAIR, is available by calling the Patent Electronic Business Center at 1-866-217-9197.

Office of Data Managment, Application Assistance Unit (571) 272-4000, or (571) 272-4200, or 1-888-786-0101

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

APPLICATION NUMBER	FILING or 371(c) DATE	GRP ART UNIT	FIL FEE REC'D	ATTY.DOCKET.NO	TOT CLAIMS	IND CLAIMS
11/709,742	02/23/2007	1637	1410	001107.00638	48	5

CONFIRMATION NO. 3875

UPDATED FILING RECEIPT

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

OC00000030559412

Date Mailed: 06/20/2008

Receipt is acknowledged of this non-provisional patent application. The application will be taken up for examination in due course. Applicant will be notified as to the results of the examination. Any correspondence concerning the application must include the following identification information: the U.S. APPLICATION NUMBER, FILING DATE, NAME OF APPLICANT, and TITLE OF INVENTION. Fees transmitted by check or draft are subject to collection. Please verify the accuracy of the data presented on this receipt. **If an error is noted on this Filing Receipt, please submit a written request for a Filing Receipt Correction. Please provide a copy of this Filing Receipt with the changes noted thereon. If you received a "Notice to File Missing Parts" for this application, please submit any corrections to this Filing Receipt with your reply to the Notice. When the USPTO processes the reply to the Notice, the USPTO will generate another Filing Receipt incorporating the requested corrections**

Applicant(s)

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

Assignment For Published Patent Application

The Johns Hopkins University, Baltimore, MD

Power of Attorney: None

Domestic Priority data as claimed by applicant

This application is a CON of 10/828,295 04/21/2004 ABN
which is a DIV of 09/981,356 10/12/2001 PAT 6,753,147
which is a CON of 09/613,826 07/11/2000 PAT 6,440,706
which claims benefit of 60/146,792 08/02/1999

Foreign Applications

If Required, Foreign Filing License Granted: 03/26/2008

The country code and number of your priority application, to be used for filing abroad under the Paris Convention, is **US 11/709,742**

Projected Publication Date: 10/02/2008

Non-Publication Request: No

Early Publication Request: No
**** SMALL ENTITY ****

Title

Digital amplification

Preliminary Class

435

PROTECTING YOUR INVENTION OUTSIDE THE UNITED STATES

Since the rights granted by a U.S. patent extend only throughout the territory of the United States and have no effect in a foreign country, an inventor who wishes patent protection in another country must apply for a patent in a specific country or in regional patent offices. Applicants may wish to consider the filing of an international application under the Patent Cooperation Treaty (PCT). An international (PCT) application generally has the same effect as a regular national patent application in each PCT-member country. The PCT process **simplifies** the filing of patent applications on the same invention in member countries, but **does not result** in a grant of "an international patent" and does not eliminate the need of applicants to file additional documents and fees in countries where patent protection is desired.

Almost every country has its own patent law, and a person desiring a patent in a particular country must make an application for patent in that country in accordance with its particular laws. Since the laws of many countries differ in various respects from the patent law of the United States, applicants are advised to seek guidance from specific foreign countries to ensure that patent rights are not lost prematurely.

Applicants also are advised that in the case of inventions made in the United States, the Director of the USPTO must issue a license before applicants can apply for a patent in a foreign country. The filing of a U.S. patent application serves as a request for a foreign filing license. The application's filing receipt contains further information and guidance as to the status of applicant's license for foreign filing.

Applicants may wish to consult the USPTO booklet, "General Information Concerning Patents" (specifically, the section entitled "Treaties and Foreign Patents") for more information on timeframes and deadlines for filing foreign patent applications. The guide is available either by contacting the USPTO Contact Center at 800-786-9199, or it can be viewed on the USPTO website at http://www.uspto.gov/web/offices/pac/doc/general/index.html.

For information on preventing theft of your intellectual property (patents, trademarks and copyrights), you may wish to consult the U.S. Government website, http://www.stopfakes.gov. Part of a Department of Commerce initiative, this website includes self-help "toolkits" giving innovators guidance on how to protect intellectual property in specific countries such as China, Korea and Mexico. For questions regarding patent enforcement issues, applicants may call the U.S. Government hotline at 1-866-999-HALT (1-866-999-4158).

LICENSE FOR FOREIGN FILING UNDER

Title 35, United States Code, Section 184

Title 37, Code of Federal Regulations, 5.11 & 5.15

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The applicant has been granted a license under 35 U.S.C. 184, if the phrase "IF REQUIRED, FOREIGN FILING LICENSE GRANTED" followed by a date appears on this form. Such licenses are issued in all applications where the conditions for issuance of a license have been met, regardless of whether or not a license may be required as

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This license is to be retained by the licensee and may be used at any time on or after the effective date thereof unless it is revoked. This license is automatically transferred to any related applications(s) filed under 37 CFR 1.53(d). This license is not retroactive.

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NOT GRANTED

No license under 35 U.S.C. 184 has been granted at this time, if the phrase "IF REQUIRED, FOREIGN FILING LICENSE GRANTED" DOES NOT appear on this form. Applicant may still petition for a license under 37 CFR 5.12, if a license is desired before the expiration of 6 months from the filing date of the application. If 6 months has lapsed from the filing date of this application and the licensee has not received any indication of a secrecy order under 35 U.S.C. 181, the licensee may foreign file the application pursuant to 37 CFR 5.15(b).

PATENT

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re Application of:	)	Group Art Unit: 1637
Bert Vogelstein, et al.	)	Docket No. 001107.00638
Serial No. 11/709,742	)	Confirmation No: 3875
Filed: February 23, 2007	)	Examiner: TBA

For: DIGITAL AMPLIFICATION

RESPONSE TO NOTICE TO FILE CORRECTED APPLICATION PAPERS

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

Dear Sir:

In response to the Notice to File Corrected Application Papers, dated April 10, 2008, Applicants submit herewith seven (7) replacement drawing sheets including FIGS. 1A-5. The period for responding to the Notice to File Corrected Application Papers expired on June 10, 2008, and thus a one-month extension of time is requested.

It is believed that all Patent and Trademark Office requirements have now been fully met and it is respectfully requested that the above-identified patent application be forwarded for examination.

Please charge the fee associated with this request and Trademark to Deposit Account No. 19-0733.

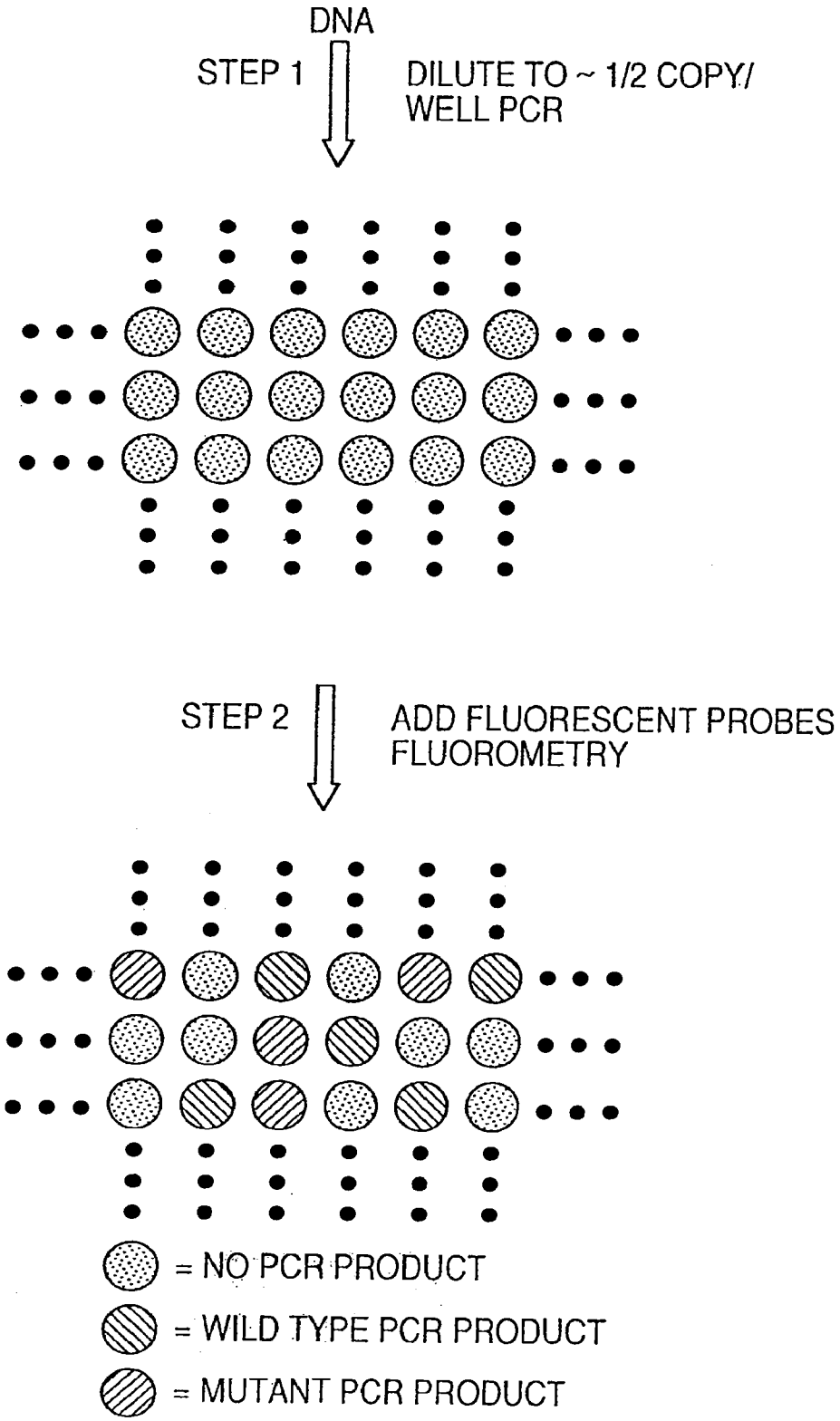
Respectfully submitted,

Dated: June 16, 2008

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

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

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

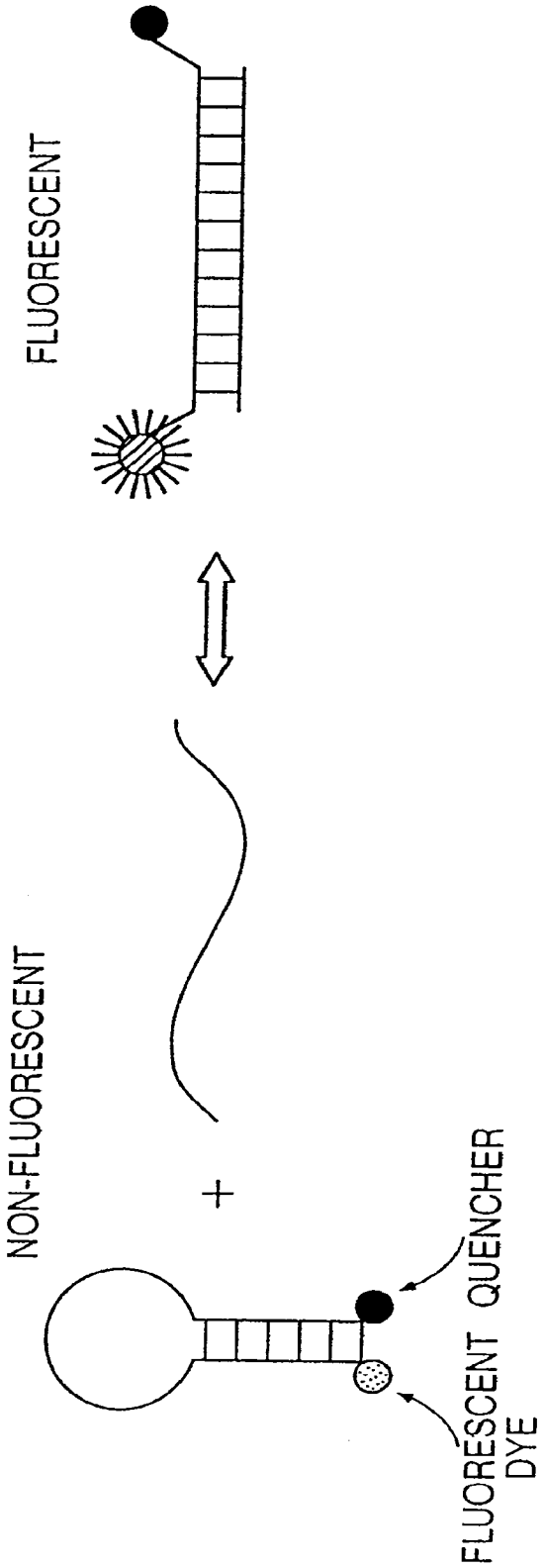


FIG. 1B

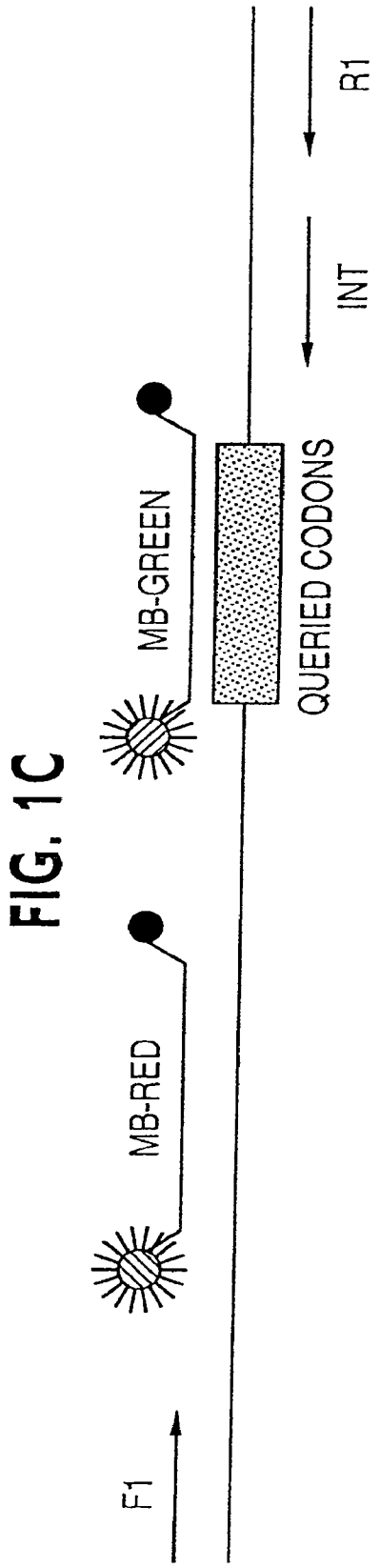
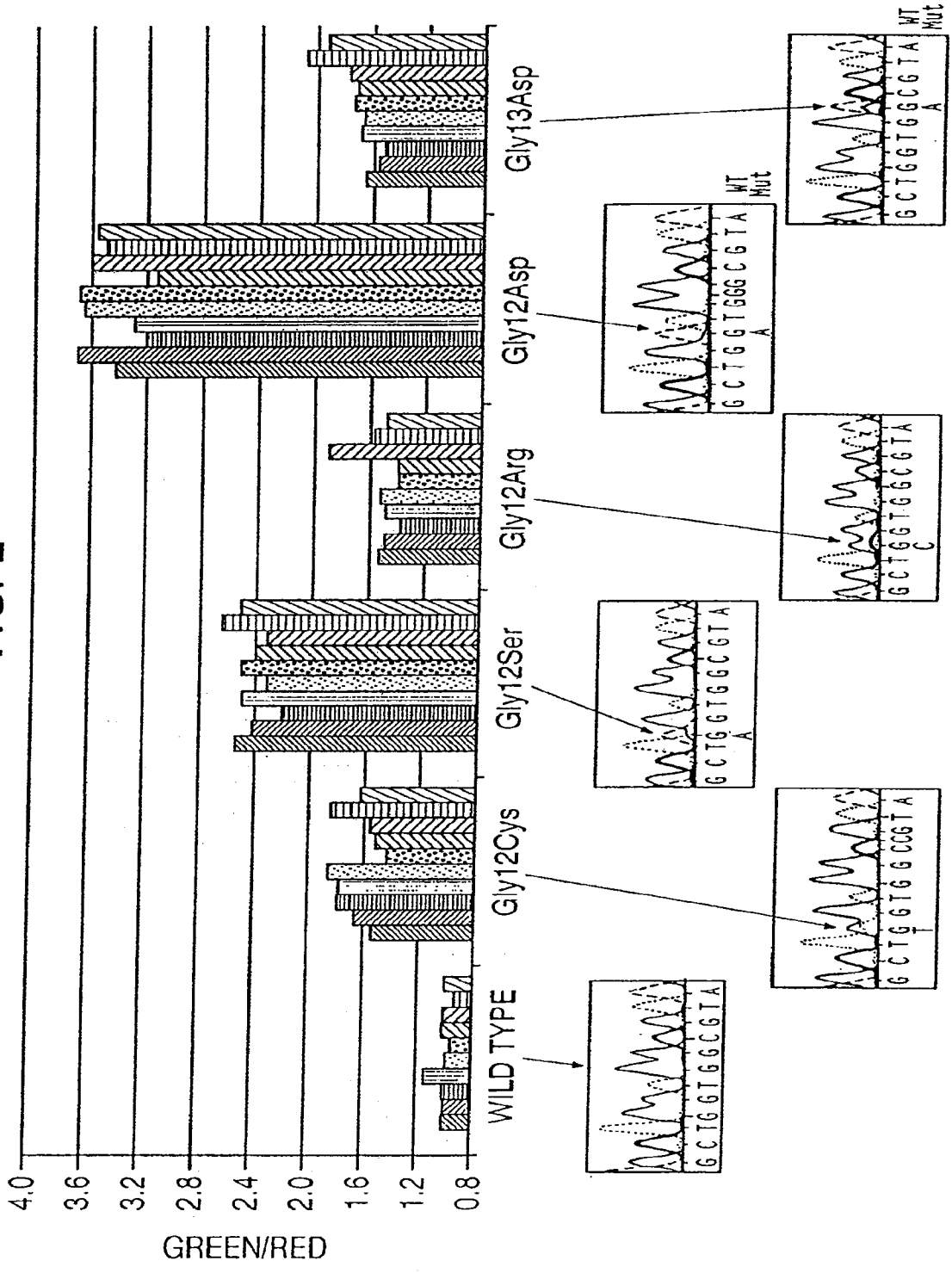


FIG. 1C

FIG. 2


Gly13Asp
Gly12Asp
Gly12Arg
Gly12Ser
Gly12Cys
WILD TYPE
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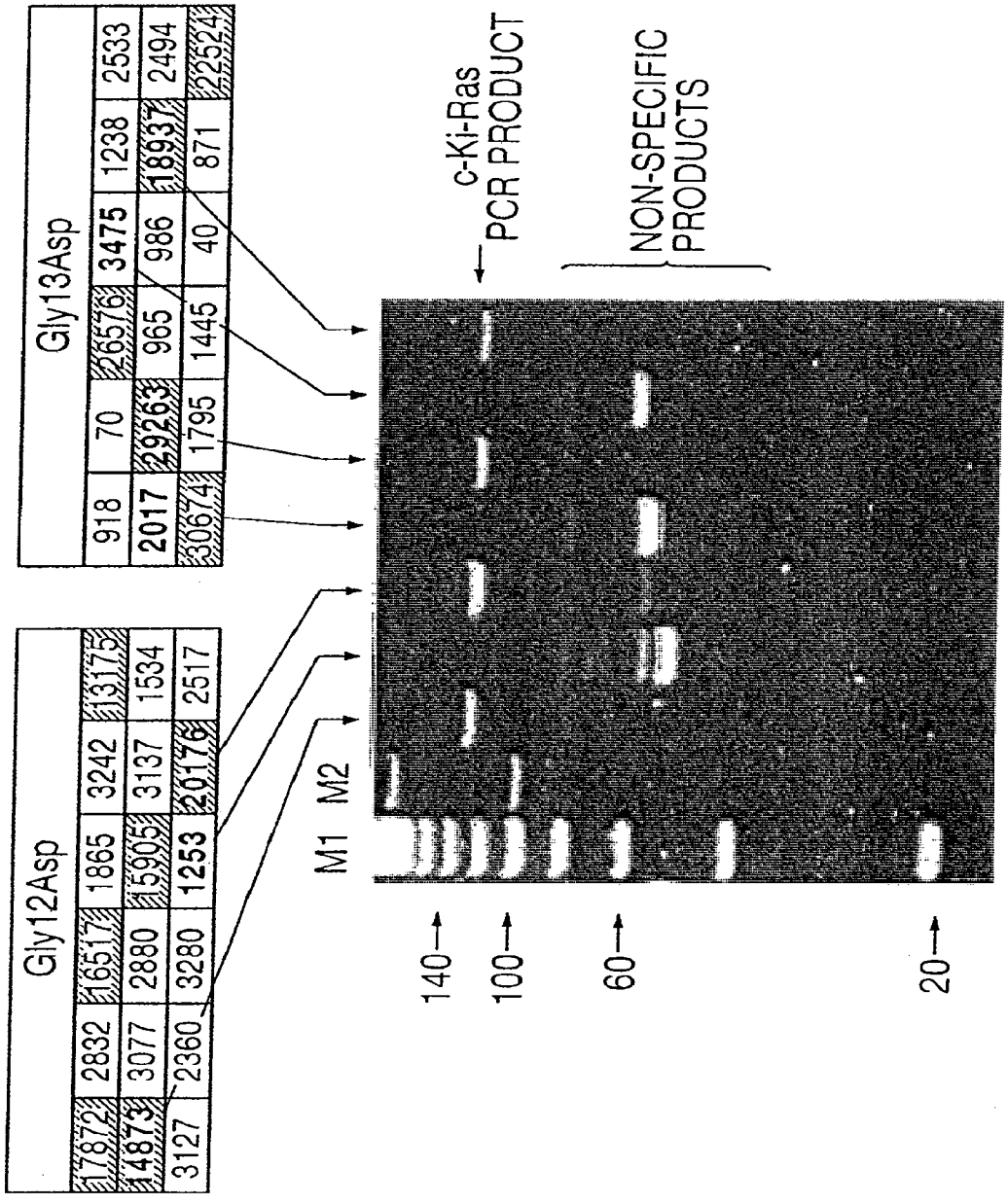
3.2
2.8
2.4
2.0
1.6
1.2
0.8
GREEN/RED
WT
Mut

FIG. 3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2017
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965
986
18937
2494
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1445
40
871
22524
c-Ki-Ras
PCR PRODUCT
NON-SPECIFIC
PRODUCTS
Gly12Asp
178722
2832
165177
1865
3242
131755
48733
3077
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M2
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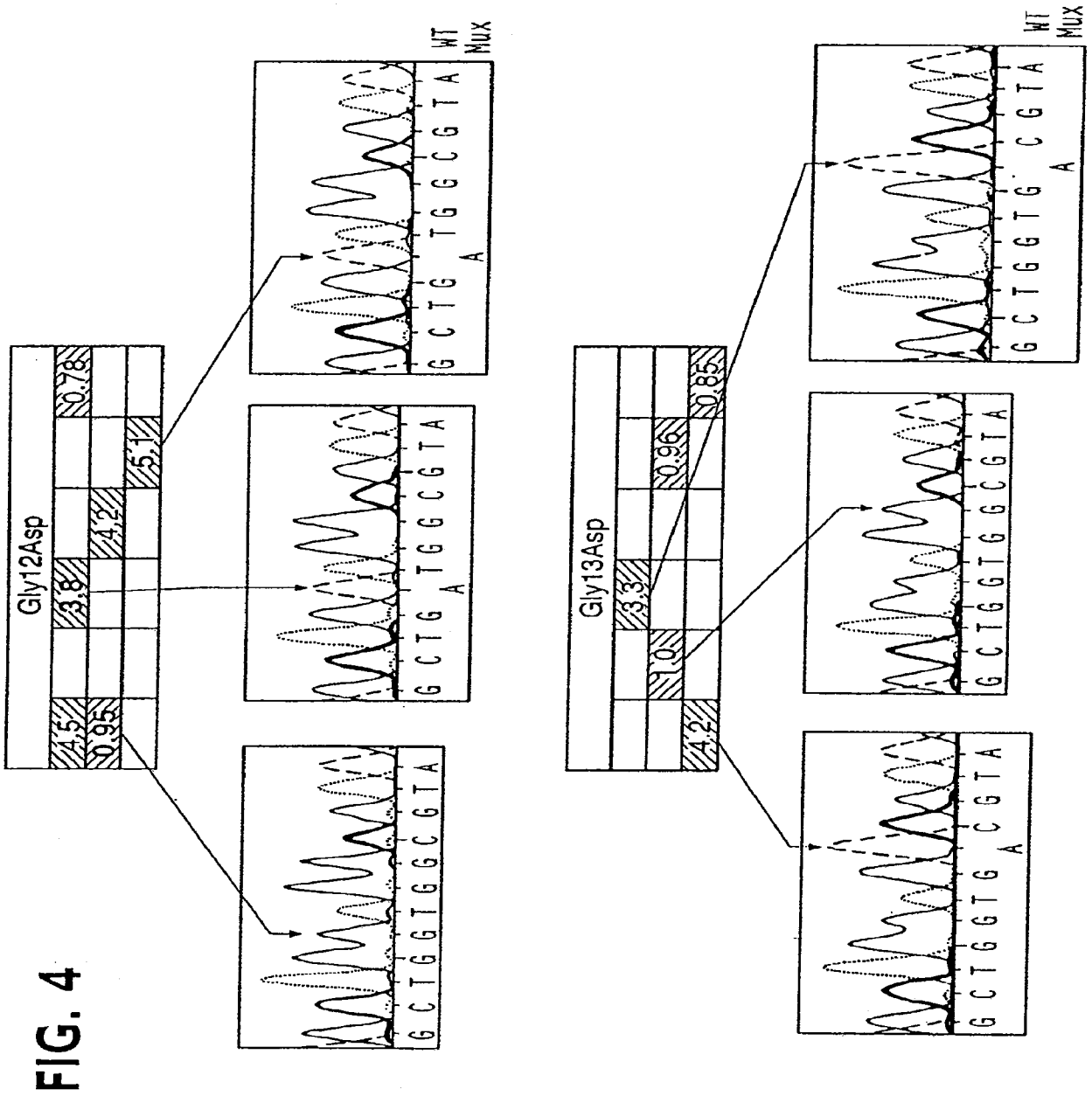
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3.3
0.96
0.85
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MUX
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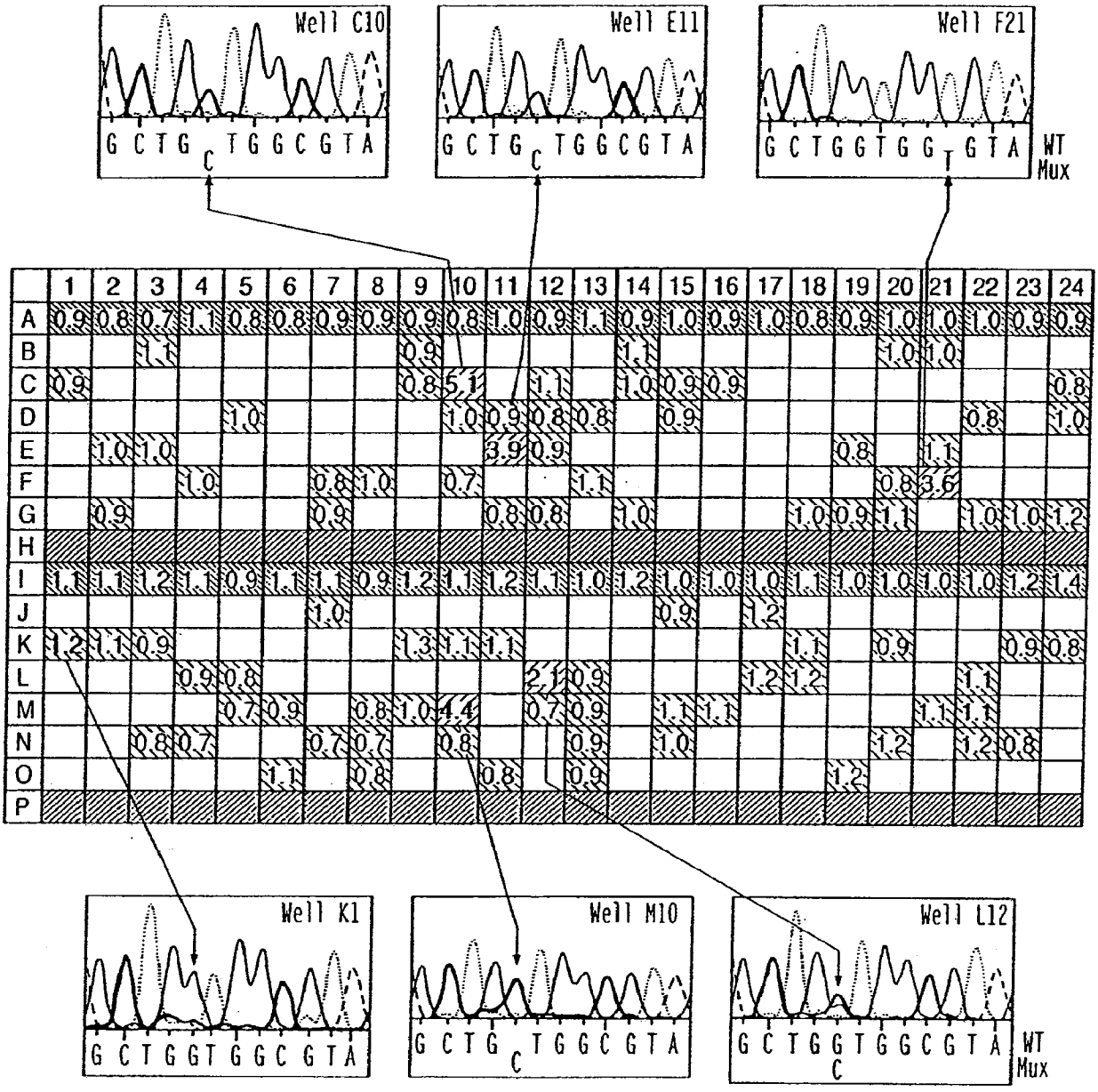
FIG. 4

FIG. 5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Well E11
G C T G C T G G C G T A
Well F21
G C T G G T G G T G T A
WT
Mux
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20 21 22 23 24
A
B
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Electronic Patent Application Fee Transmittal

Application Number:	11709742
Filing Date:	23-Feb-2007
Title of Invention:	Digital amplification
First Named Inventor/Applicant Name:	Bert Vogelstein
Filer:	Sarah Anne Kagan./Jimani Walden
Attorney Docket Number:	001107.00638

Filed as Small Entity

Utility Filing Fees

Description	Fee Code	Quantity	Amount	Sub-Total in USD($)
Basic Filing:				
Pages:				
Claims:				
Miscellaneous-Filing:				
Petition:				
Patent-Appeals-and-Interference:				
Post-Allowance-and-Post-Issuance:				
Extension-of-Time:				
Extension - 1 month with $0 paid	2251	1	60	60

Description	Fee Code	Quantity	Amount	Sub-Total in USD($)
Miscellaneous:				
			Total in USD ($)	60

Electronic Acknowledgement Receipt

EFS ID:	3460904
Application Number:	11709742
International Application Number:	
Confirmation Number:	3875
Title of Invention:	Digital amplification
First Named Inventor/Applicant Name:	Bert Vogelstein
Customer Number:	22907
Filer:	Sarah Anne Kagan./Jimani Walden
Filer Authorized By:	Sarah Anne Kagan.
Attorney Docket Number:	001107.00638
Receipt Date:	16-JUN-2008
Filing Date:	23-FEB-2007
Time Stamp:	14:18:52
Application Type:	Utility under 35 USC 111(a)

Payment information:

Submitted with Payment	yes
Payment Type	Deposit Account
Payment was successfully received in RAM	$60
RAM confirmation Number	10224
Deposit Account	190733
Authorized User	

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

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

File Listing:

Document Number	Document Description	File Name	File Size(Bytes) /Message Digest	Multi Part /.zip	Pages (if appl.)
1	Applicant Response to Pre-Exam Formalities Notice	response.PDF	34454 a09d06835626e8b6ef9bccedd7a91d1cb8761e1b	no	1
Warnings:					
Information:					
2	Drawings-only black and white line drawings	replacementsheets.PDF	445015 f9f6d3a8a1cc0de918eeef90a11c3daec2d50344	no	7
Warnings:					
Information:					
3	Fee Worksheet (PTO-06)	fee-info.pdf	8124 e45720aed4386f3187b373642036170d23322daa	no	2
Warnings:					
Information:					
		Total Files Size (in bytes):	487593		

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.

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

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

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

Electronic Acknowledgement Receipt

EFS ID:	3460904
Application Number:	11709742
International Application Number:	
Confirmation Number:	3875
Title of Invention:	Digital amplification
First Named Inventor/Applicant Name:	Bert Vogelstein
Customer Number:	22907
Filer:	Sarah Anne Kagan./Jimani Walden
Filer Authorized By:	Sarah Anne Kagan.
Attorney Docket Number:	001107.00638
Receipt Date:	16-JUN-2008
Filing Date:	23-FEB-2007
Time Stamp:	14:18:52
Application Type:	Utility under 35 USC 111(a)

Payment information:

Submitted with Payment	yes
Payment Type	Deposit Account
Payment was successfully received in RAM	$60
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Authorized User	

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

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

File Listing:

Document Number	Document Description	File Name	File Size(Bytes) /Message Digest	Multi Part /.zip	Pages (if appl.)
1	Applicant Response to Pre-Exam Formalities Notice	response.PDF	34454 a09d06835626e8b6ef9bccedd7a91d1cb8761e1b	no	1
Warnings:					
Information:					
2	Drawings-only black and white line drawings	replacementsheets.PDF	445015 f9f6d3a8a1cc0de918eeef90a11c3daec2d50344	no	7
Warnings:					
Information:					
3	Fee Worksheet (PTO-06)	fee-info.pdf	8124 e45720aed4386f3187b373642036170d23322daa	no	2
Warnings:					
Information:					
		Total Files Size (in bytes):	487593		

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
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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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United States Patent and Trademark Office
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P.O. Box 1450
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APPLICATION NUMBER	FILING OR 371(C) DATE	FIRST NAMED APPLICANT	ATTY. DOCKET NO./TITLE
11/709,742	02/23/2007	Bert Vogelstein	001107.00638

CONFIRMATION NO. 3875

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

FORMALITIES LETTER

OC000000029298715

Date Mailed: 04/10/2008

NOTICE TO FILE CORRECTED APPLICATION PAPERS

Filing Date Granted

An application number and filing date have been accorded to this application. The application is informal since it does not comply with the regulations for the reason(s) indicated below. Applicant is given TWO MONTHS from the date of this Notice within which to correct the informalities indicated below. Extensions of time may be obtained by filing a petition accompanied by the extension fee under the provisions of 37 CFR 1.136(a).

The required item(s) identified below must be timely submitted to avoid abandonment:

- Replacement drawings in compliance with 37 CFR 1.84 and 37 CFR 1.121(d) are required. The drawings submitted are not acceptable because:
  - The drawings must be reasonably free from erasures and must be free from alterations, overwriting, interlineations, folds, and copy marks. See Figure(s) ALL.
  - The drawings have a line quality that is too light to be reproduced (weight of all lines and letters must be heavy enough to permit adequate reproduction) or text that is illegible (reference characters, sheet numbers, and view numbers must be plain and legible) see 37 CFR 1.84(l) and (p)(1)); See Figure(s) 5.

Applicant is cautioned that correction of the above items may cause the specification and drawings page count to exceed 100 pages. If the specification and drawings exceed 100 pages, applicant will need to submit the required application size fee.

Replies should be mailed to:

Mail Stop Missing Parts
Commissioner for Patents
P.O. Box 1450
Alexandria VA 22313-1450

Registered users of EFS-Web may alternatively submit their reply to this notice via EFS-Web.
https://sportal.uspto.gov/authenticate/AuthenticateUserLocalEPF.html

For more information about EFS-Web please call the USPTO Electronic Business Center at **1-866-217-9197** or visit our website at http://www.uspto.gov/ebc.

If you are not using EFS-Web to submit your reply, you must include a copy of this notice.

/nfissha/

Office of Data Management, Application Assistance Unit (571) 272-4000, or (571) 272-4200, or 1-888-786-0101

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

APPLICATION NUMBER	FILING or 371(c) DATE	GRP ART UNIT	FIL FEE REC'D	ATTY.DOCKET.NO	TOT CLAIMS	IND CLAIMS
11/709,742	02/23/2007	1637	1410	001107.00638	48	5

CONFIRMATION NO. 3875

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

FILING RECEIPT

OC00000029298714

Date Mailed: 04/10/2008

Receipt is acknowledged of this non-provisional patent application. The application will be taken up for examination in due course. Applicant will be notified as to the results of the examination. Any correspondence concerning the application must include the following identification information: the U.S. APPLICATION NUMBER, FILING DATE, NAME OF APPLICANT, and TITLE OF INVENTION. Fees transmitted by check or draft are subject to collection. Please verify the accuracy of the data presented on this receipt. **If an error is noted on this Filing Receipt, please write to the Office of Initial Patent Examination's Filing Receipt Corrections. Please provide a copy of this Filing Receipt with the changes noted thereon. If you received a "Notice to File Missing Parts" for this application, please submit any corrections to this Filing Receipt with your reply to the Notice. When the USPTO processes the reply to the Notice, the USPTO will generate another Filing Receipt incorporating the requested corrections**

Applicant(s)

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

Assignment For Published Patent Application

The Johns Hopkins University, Baltimore, MD

Power of Attorney: None

Domestic Priority data as claimed by applicant

This application is a CON of 10/828,295 04/21/2004 ABN
which is a DIV of 09/981,356 10/12/2001 PAT 6,753,147
which is a CON of 09/613,826 07/11/2000 PAT 6,440,706
which claims benefit of 60/146,792 08/02/1999

Foreign Applications

If Required, Foreign Filing License Granted: 03/26/2008

The country code and number of your priority application, to be used for filing abroad under the Paris Convention, is **US 11/709,742**

Projected Publication Date: To Be Determined - pending completion of Corrected Papers

Non-Publication Request: No

Early Publication Request: No
**** SMALL ENTITY ****

Title

Digital amplification

Preliminary Class

435

PROTECTING YOUR INVENTION OUTSIDE THE UNITED STATES

Since the rights granted by a U.S. patent extend only throughout the territory of the United States and have no effect in a foreign country, an inventor who wishes patent protection in another country must apply for a patent in a specific country or in regional patent offices. Applicants may wish to consider the filing of an international application under the Patent Cooperation Treaty (PCT). An international (PCT) application generally has the same effect as a regular national patent application in each PCT-member country. The PCT process **simplifies** the filing of patent applications on the same invention in member countries, but **does not result** in a grant of "an international patent" and does not eliminate the need of applicants to file additional documents and fees in countries where patent protection is desired.

Almost every country has its own patent law, and a person desiring a patent in a particular country must make an application for patent in that country in accordance with its particular laws. Since the laws of many countries differ in various respects from the patent law of the United States, applicants are advised to seek guidance from specific foreign countries to ensure that patent rights are not lost prematurely.

Applicants also are advised that in the case of inventions made in the United States, the Director of the USPTO must issue a license before applicants can apply for a patent in a foreign country. The filing of a U.S. patent application serves as a request for a foreign filing license. The application's filing receipt contains further information and guidance as to the status of applicant's license for foreign filing.

Applicants may wish to consult the USPTO booklet, "General Information Concerning Patents" (specifically, the section entitled "Treaties and Foreign Patents") for more information on timeframes and deadlines for filing foreign patent applications. The guide is available either by contacting the USPTO Contact Center at 800-786-9199, or it can be viewed on the USPTO website at http://www.uspto.gov/web/offices/pac/doc/general/index.html.

For information on preventing theft of your intellectual property (patents, trademarks and copyrights), you may wish to consult the U.S. Government website, http://www.stopfakes.gov. Part of a Department of Commerce initiative, this website includes self-help "toolkits" giving innovators guidance on how to protect intellectual property in specific countries such as China, Korea and Mexico. For questions regarding patent enforcement issues, applicants may call the U.S. Government hotline at 1-866-999-HALT (1-866-999-4158).

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Title 35, United States Code, Section 184

Title 37, Code of Federal Regulations, 5.11 & 5.15

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PATENT APPLICATION SERIAL NO. 11709742

U.S. DEPARTMENT OF COMMERCE
PATENT AND TRADEMARK OFFICE
FEE RECORD SHEET

02/26/2007 SSITHIB1 00000051 190733 11709742

01 FC:2011 150.00 DA
02 FC:2111 250.00 DA
03 FC:2311 100.00 DA
04 FC:2202 200.00 DA

03/26/2008 MTEKLEMI 00000044 190733 11709742

01 FC:2201 210.00 DA
02 FC:2202 500.00 DA

PTO-1556
(5/87)

*U.S. Government Printing Office: 2002 — 460-247/60043

RAW SEQUENCE LISTING

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Application Serial Number: 11/709,742
Source: OIPE
Date Processed by SCORE: 3/12/08

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SEQUENCE LISTING

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Kinzler, Kenneth W.

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DFW

PATENT

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re Application of	)	Prior Group Art Unit: 1637
	)	
Bert VOGELSTEIN et al	)	Prior Examiner: M. Baughman
	)	
Serial No. 11/709,742	)	Confirmation No. TBA
	)	
Filed: February 22, 2007	)	Atty. Dkt. No. 001107.00638

For: DIGITAL AMPLIFICATION

PRELIMINARY AMENDMENT

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

Sir:

Applicants respectfully request that the following claim set be entered prior to examination on the merits. Please charge any necessary additional fee to our deposit account no. 19-0733.

CLAIMS

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

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

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

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

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

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

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

4. (original) The method of claim 1 wherein the step of diluting is performed until all of the assay samples yield an amplification product when subjected to a polymerase chain reaction and

each assay sample contains less than 100 nucleic acid template molecules containing a reference genetic sequence.

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

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

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

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

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

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

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

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

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

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

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

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

17. (original) The method of claim 13 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. (New) 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. (New) The method of claim 29 wherein the step of amplifying employs **real-time polymerase chain reactions**.

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

Remarks

Claim 29 recites a method in which a sample from blood is tested to determine a ratio of two genetic sequences. This is supported at page 11, lines 3-6:

> 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.

Support for claims 36, 39 and 48 (sample from blood) is similar.

Claim 30, dependent on claim 29, recites real-time PCR. This is supported at page 9, line 6. Support for claims 37 and 40 and 46 (real-time PCR) is similar.

Claims 31 recites dual-labeled fluorogenic probes. This recitation is supported at page 12, lines 8-9. Support for claims 38 and 41 and 47 (probes) is similar.

Claim 32 recites identification of an allelic imbalance. This is supported at page 9, lines 9-11 and at the sentence spanning pages 10 and 11. Support for claims 35, 42, and 45 is similar.

Claims 33, 43, and 45 recite non-polymorphic markers. Such markers are supported at Table 1, last line.

Claim 34 recites that the two compared genetic sequences are located on distinct chromosomes. This is supported at Table 1, last line. Claim 44 is similarly supported.

No new matter is added to the application by these new claims.

Respectfully submitted,

Date: February 14, 2007

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

Banner & Witcoff, Ltd.
Customer No. 22907

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

PATENT APPLICATION FEE DETERMINATION RECORD

Substitute for Form PTO-875

Application or Docket Number	Filing Date	☐ To be Mailed
11/709,742	02/23/2007	

APPLICATION AS FILED – PART I

FOR	(Column 1) NUMBER FILED	(Column 2) NUMBER EXTRA	SMALL ENTITY ☒ RATE ($)	SMALL ENTITY ☒ FEE ($)	OR	OTHER THAN SMALL ENTITY RATE ($)	OTHER THAN SMALL ENTITY FEE ($)
☐ BASIC FEE (37 CFR 1.16(a), (b), or (c))	N/A	N/A	N/A			N/A	
☐ SEARCH FEE (37 CFR 1.16(k), (i), or (m))	N/A	N/A	N/A			N/A	
☐ EXAMINATION FEE (37 CFR 1.16(o), (p), or (q))	N/A	N/A	N/A			N/A	
TOTAL CLAIMS (37 CFR 1.16(i))	minus 20 =	*	X $ =		OR	X $ =	
INDEPENDENT CLAIMS (37 CFR 1.16(h))	minus 3 =	*	X $ =			X $ =	
☐ APPLICATION SIZE FEE (37 CFR 1.16(s))	If the specification and drawings exceed 100 sheets of paper, the application size fee due is $250 ($125 for small entity) for each additional 50 sheets or fraction thereof. See 35 U.S.C. 41(a)(1)(G) and 37 CFR 1.16(s).						
☐ MULTIPLE DEPENDENT CLAIM PRESENT (37 CFR 1.16(j))							
* If the difference in column 1 is less than zero, enter "0" in column 2.			TOTAL			TOTAL	

APPLICATION AS AMENDED – PART II

AMENDMENT 02/14/2008

	(Column 1) CLAIMS REMAINING AFTER AMENDMENT		(Column 2) HIGHEST NUMBER PREVIOUSLY PAID FOR	(Column 3) PRESENT EXTRA	SMALL ENTITY RATE ($)	SMALL ENTITY ADDITIONAL FEE ($)	OR	OTHER THAN SMALL ENTITY RATE ($)	OTHER THAN SMALL ENTITY ADDITIONAL FEE ($)
Total (37 CFR 1.16(i))	* 48	Minus	** 48	= 0	X $25 =	0	OR	X $ =	
Independent (37 CFR 1.16(h))	* 5	Minus	***5	= 0	X $105 =	0	OR	X $ =	
☐ Application Size Fee (37 CFR 1.16(s))									
☐ FIRST PRESENTATION OF MULTIPLE DEPENDENT CLAIM (37 CFR 1.16(j))							OR		
					TOTAL ADD'L FEE	0	OR	TOTAL ADD'L FEE	

AMENDMENT

	(Column 1) CLAIMS REMAINING AFTER AMENDMENT		(Column 2) HIGHEST NUMBER PREVIOUSLY PAID FOR	(Column 3) PRESENT EXTRA	RATE ($)	ADDITIONAL FEE ($)	OR	RATE ($)	ADDITIONAL FEE ($)
Total (37 CFR 1.16(i))	*	Minus	**	=	X $ =		OR	X $ =	
Independent (37 CFR 1.16(h))	*	Minus	***	=	X $ =		OR	X $ =	
☐ Application Size Fee (37 CFR 1.16(s))									
☐ FIRST PRESENTATION OF MULTIPLE DEPENDENT CLAIM (37 CFR 1.16(j))							OR		
					TOTAL ADD'L FEE		OR	TOTAL ADD'L FEE	

* If the entry in column 1 is less than the entry in column 2, write "0" in column 3.

** If the "Highest Number Previously Paid For" IN THIS SPACE is less than 20, enter "20".

*** If the "Highest Number Previously Paid For" IN THIS SPACE is less than 3, enter "3".

The "Highest Number Previously Paid For" (Total or Independent) is the highest number found in the appropriate box in column 1.

Legal Instrument Examiner:
/ANDREW JAMES JR/

U.S. PTO
11/709742
02/23/2007

PTO/SB/05 (09-04)
Approved for use through 07/31/2006. OMB 0651-0032
U.S. Patent and Trademark Office. U.S. DEPARTMENT OF COMMERCE

UTILITY PATENT APPLICATION TRANSMITTAL

(Only for new nonprovisional applications under 37 C.F.R. 1.53(b))

Attorney Docket No.	001107.00638
First Inventor	Bert VOLGESTEIN et al.
Title	Digital Amplification
Express Mail Label No.	

APPLICATION ELEMENTS

See MPEP chapter 600 concerning utility patent application contents.

1. ☒ **Fee Transmittal Form (e.g., PTO/SB/17)**
   (Submit an original and a duplicate for fee processing)
2. ☒ **Applicant claims small entity status.** See 37 CFR 1.27.
3. ☒ **Specification** [*Total Pages* 25]
   Both the claims and abstract must start on a new page
   (For information on the preferred arrangement, see MPEP 608.01(a))
4. ☒ **Drawing(s)** (*35 U.S.C.113*) [*Total Sheets* 7]
   ☒ Formal ☐ Informal
5. **Oath or Declaration** [*Total Sheets* 2]
   a. ☐ Newly executed (original or copy)
   b. ☒ Copy from a prior application (37 CFR 1.63 (d))
   (for a continuation/divisional with Box 18 completed)
   i. ☐ DELETION OF INVENTOR(S)
   Signed statement attached deleting inventor(s) named in the prior application, see 37 CFR 1.63(d)(2) and 1.33(b).
6. ☒ **Application Data Sheet.** See 37 CFR 1.76
7. ☐ **CD-ROM or CD-R** in duplicate, large table or Computer Program *(Appendix)*
   ☐ Landscape Table on CD
8. **Nucleotide and/or Amino Acid Sequence Submission**
   (if applicable, items a.-c. are required)
   a. ☐ Computer Readable Form (CRF)
   b. Specification Sequence Listing on:
   i. ☐ CD-ROM or CD-R (2 copies); or
   ii. ☒ Paper
   c. ☒ Statements verifying identity of above copies

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

ACCOMPANYING APPLICATIONS PARTS

9. ☐ **Assignment Papers** (cover sheet & document(s))
   Name of Assignee _____
10. ☐ **37 C.F.R. 3.73(b) Statement** ☐ **Power of Attorney**
    (when there is an assignee)
11. ☐ **English Translation Document** *(if applicable)*
12. ☒ **Information Disclosure Statement** (PTO/SB/08 or PTO-1449)
    ☐ Copies of citations attached
13. ☐ **Preliminary Amendment**
14. ☒ **Return Receipt Postcard** (MPEP 503)
    (Should be specifically itemized)
15. ☐ **Certified Copy of Priority Document(s)**
    (if foreign priority is claimed)
16. ☐ **Nonpublication Request** under 35 U.S.C. 122(b)(2)(B)(i). Applicant must attach form PTO/SB/35 or its equivalent.
17. ☐ Other: _____

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

☒ Continuation ☐ Divisional ☐ Continuation-in-part (CIP) of prior application No: 10 / 828,295

Prior application information: *Examiner* M. Baughman *Art Unit:* 1637

19. CORRESPONDENCE ADDRESS

☒ *Customer Number* **22907** ***OR*** ☐ *Correspondence address below*

Name					
Address					
City		*State*		*Zip Code*	
Country		*Telephone*		*Fax*	

Signature	/Sarah A. Kagan/	Date	February 23, 2007
Name (Print/Type)	Sarah A. Kagan	*Registration No. (Attorney/Agent)*	32,141

PTO/SB/17 (12-04v2)
Approved for use through 07/31/2006. OMB 0651-0032
U.S. Patent and Trademark Office: U.S. DEPARTMENT OF COMMERCE

Effective on 12/08/2004.
Fees pursuant to the Consolidated Appropriations Act, 2005 (H.R. 4818).

FEE TRANSMITTAL for FY 2005

☒ Applicant claims small entity status. See 37 CFR 1.27

TOTAL AMOUNT OF PAYMENT	($) 700

Complete If Known	
Application Number	TBD
Filing Date	February 23 , 2007
First Named Inventor	Bert VOGELSTEIN et al.
Examiner Name	TBD
Art Unit	TBD
Attorney Docket No.	001107.00638

METHOD OF PAYMENT (check all that apply)

☐ Check ☐ Credit Card ☐ Money Order ☐ None ☐ Other (please identify) : _____

☒ Deposit Account Deposit Account Number: 19-0733 Deposit Account Name: Banner & Witcoff, LTD.

For the above-identified deposit account, the Director is hereby authorized to: (check all that apply)

☒ Charge fee(s) indicated below ☐ Charge fee(s) indicated below, **except for the filing fee**

☒ Charge any additional fee(s) or underpayments of fee(s) Under 37 CFR 1.16 and 1.17 ☒ Credit any overpayments

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.

FEE CALCULATION

1. BASIC FILING, SEARCH, AND EXAMINATION FEES

	FILING FEES	Small Entity	SEARCH FEES	Small Entity	EXAMINATION FEES	Small Entity	
Application Type	Fee ($)	Fee($)	Fee($)	Fee($)	Fee($)	Fee($)	Fees Paid ($)
Utility	300	150	500	250	200	100	500.00
Design	200	100	100	50	130	65	____
Plant	200	100	300	150	160	80	____
Reissue	300	150	500	250	600	300	____
Provisional	200	100	0	0	0	0	____

2. EXCESS CLAIM FEES

Fee Description	Fee ($)	Small Entity Fee ($)
Each claim over 20 (including Reissues)	50	25
Each independent claim over 3 (including Reissues)	200	100
Multiple dependent claims	360	180

Total Claims		Extra Claims		Fee($)		Fee Paid ($)
28	- 20 or HP=	8	x	25	=	200.00

HP = highest number of total claims paid for, if greater than 20.

Indep. Claims		Extra Claims		Fee($)		Fee Paid ($)
1	- 3 or HP=	0	x	0	=	0

HP = highest number of independent claims paid for, if greater than 3.

Multiple Dependent Claims	Fee ($)	Fee Paid ($)
	____	____

3. APPLICATION SIZE FEE

If the specification and drawings exceed 100 sheets of paper (excluding electronically filed sequence or computer listings under 37 CFR 1.52(e)), 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).

Total Sheets		Extra Sheets		Number of each additional 50 or fraction thereof		Fee ($)	Fee Paid ($)
____	- 100 =	____	/ 50 =	____	(round up to a whole number) x		= ____

4. OTHER FEE(S)

	Fees Paid ($)
Non-English Specification, $130 fee (no small entity discount)	____
Other (e.g., late filing surcharge) : _____	____

SUBMITTED BY

Signature		Registration No. (Attorney/Agent) 32,141	Telephone (202) 824-3000
Name (Print/Type)	/Sarah A. Kagan/ Sarah A. Kagan		Date February 23, 2007

DIGITAL AMPLIFICATION

This application is a continuation of U.S. Application Serial Number 10/828,295 filed April 21, 2004, which is a divisional of U.S. Application Serial Number 09/981,356 filed October 12, 2001, now US Patent 6,753,147, which is a continuation of U.S. Application Serial Number 09/613,826 filed July 11, 2000, now U.S. Patent 6,440,706, which claims the benefit of provisional U.S. Application Serial Number 60/146,792, filed August 2, 1999. The disclosure of all priority applications is expressly incorporated herein.

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.

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

According to another embodiment of the invention, a molecular beacon probe is provided. It comprises an oligonucleotide with a stem-loop structure having a photoluminescent dye at one of the 5' or 3' ends and a quenching

agent at the opposite 5' or 3' end. The loop consists of 16 base pairs which has a T_m of 50-51□C. The stem consists of 4 base pairs having a sequence 5'-CACG-3'.

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

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

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

BRIEF DESCRIPTION OF THE DRAWINGS

FIG. 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 ~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 +/- 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.

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 amplifable template molecules. Desirably each assay sample prior to amplification will contain less than a hundred or less than ten template molecules.

Digital amplification can be used to detect mutations present at relatively low levels in the samples to be analyzed. The limit of detection is defined by the number of wells that can be analyzed and the intrinsic mutation rate of the polymerase used for amplification. 384 well PCR plates are commercially available and 1536 well plates are on the horizon, theoretically allowing sensitivities for mutation detection at the ~0.1% level. It is also possible that Digital Amplification can be performed in microarray format, potentially increasing the sensitivity by another order of magnitude. This sensitivity may ultimately be limited by polymerase errors. The effective error rate in PCR as performed under our conditions was 1.1%, i.e., four out of 351 PCR products derived from WT DNA sequence appeared to contain a mutation by RED/GREEN ratio criteria. However, any individual mutation (such as a

G to T transversion at the second position of codon 12 of *c-Ki-Ras*), are expected to occur in < 1 in 50 of these polymerase-generated mutants (there are at least 50 base substitutions within or surrounding codons 12 and 13 that should yield high RED/GREEN ratios). Determining the sequence of the putative mutants in the positive wells, by direct sequencing as performed here or by any of the other techniques, provides unequivocal validation of a prospective mutation: a significant fraction of the mutations found in individual wells should be identical if the mutation occurred *in vivo*. Significance can be established through rigorous statistical analysis, as positive signals should be distributed according to Poisson probabilities. Moreover, the error rate in particular Digital Amplification experiments can be precisely determined through performance of Digital Amplification on DNA templates from normal cells.

Digital Amplification is as easily applied to RT-PCR products generated from RNA templates as it is to genomic DNA. For example, the fraction of alternatively spliced or mutant transcripts from a gene can be easily determined using photoluminescent probes specific for each of the PCR products generated. Similarly, Digital Amplification can be used to quantitate relative levels of gene expression within an RNA population. For this amplification, each well would contain primers which are used to amplify a reference transcript expressed constitutively as well as primers specific for the experimental transcript. One photoluminescent probe would then be used to detect PCR products from the reference transcript and a second photoluminescent probe used for the test transcript. The number of wells in which the test transcript is amplified divided by the number of wells in which the reference transcript is amplified provides a quantitative measure of gene expression. Another group of examples involves the investigations of allelic status when two mutations are observed upon sequence analysis of a standard DNA sample. To distinguish whether one variant is present in each allele (*vs.*

both occurring in one allele), cloning of PCR products is generally performed. The approach described here would simplify the analysis by eliminating the need for cloning. Other potential applications of Digital Amplification are listed in Table 1. When the goal is the quantitation of the proportion of two relatively common alleles or transcripts rather than the detection of rare alleles, techniques such as those employing TaqMan and real time PCR provide an excellent alternative to use of molecular beacons. Advantages of real time PCR methods include their simplicity and the ability to analyze multiple samples simultaneously. However, Digital Amplification may prove useful for these applications when the expected differences are small, (*e.g.*, only ~2-fold, such as occurs with allelic imbalances.)

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

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

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

and 0.6, more preferably to between 0.3 and 0.5 of the assay samples yielding an amplification product.

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

The number of different situations in which the digital amplification method will find application is large. Some of these are listed in Table 1. As shown in the examples, the method can be used to find a tumor mutation in a population of cells which is not purely tumor cells. As described in the examples, a probe for a particular mutation need not be used, but diminution in binding to a wild-type probe can be used as an indicator of the presence of one or more mutations. Chromosomal translocations which are characteristic of leukemias or lymphomas can be detected as a measure of the efficacy of therapy. Gene amplifications are characteristic of certain disease states. These can be measured using digital amplification. Alternatively spliced forms of a transcript can be detected and quantitated relative to other forms of the transcript using digital amplification on cDNA made from mRNA. Similarly, using cDNA made from mRNA one can determine relative levels of transcription of two different genes. One can use digital amplification to distinguish between a situation where one allele carries two mutations and one mutation is carried on each of two alleles in an individual. Allelic imbalances

often result from a disease state. These can be detected using digital amplification.

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

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

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

invention to any particular form of signal transduction. Ratiometric measurements of photoluminescence intensity can include the measurement of changes in intensity, photoluminescence lifetimes, or even polarization (anisotropy).

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

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

EXAMPLE 1

Step 1: PCR amplifications. The optimal conditions for PCR described in this section were determined by varying the parameters described in the Results. PCR was performed in 7 ul volumes in 96 well polypropylene PCR plates (RPI). The composition of the reactions was: 67 mM Tris, pH 8.8, 16.6 mM NH_4SO_4, 6.7 mM $MgCl_2$, 10 mM β-mercaptoethanol, 1 mM dATP, 1 mM dCTP, 1 mM dGTP, 1 mM TTP, 6% DMSO, 1 uM primer F1, 1 uM primer R1, 0.05 units/ul Platinum Taq polymerase (Life Technologies, Inc.), and "one-half genome equivalent" of DNA. To determine the amount of DNA corresponding to one-half genome equivalent, DNA samples were serially diluted and tested via PCR. The amount that yielded amplification products in half the wells, usually ~1 pg of total DNA, was defined as "one-half genome equivalent" and used in each well of subsequent Digital Amplification experiments. Fifty ul light mineral oil (Sigma M-3516) was added to each well

and reactions performed in a HybAid Thermal cycler at the following temperatures: denaturation at 94° for one min; 60 cycles of 94° for 15 sec, 55° for 15 sec., 70° for 15 seconds; 70° for five minutes. Reactions were read immediately or stored at room temperature for up to 36 hours before fluorescence analysis.

EXAMPLE 2

Step 2: Fluorescence analysis. 3.5 ul of a solution with the following composition was added to each well: 67 mM Tris, pH 8.8, 16.6 mM NH_4SO_4, 6.7 mM $MgCl_2$, 10 mM β-mercaptoethanol, 1 mM dATP, 1 mM dCTP, 1 mM dGTP, 1 mM TTP, 6% DMSO, 5 uM primer INT, 1 uM MB-GREEN, 1 uM MB-RED, 0.1 units/ul Platinum Taq polymerase. The plates were centrifuged for 20 seconds at 6000 g and fluorescence read at excitation/emission wavelengths of 485 nm/530 nm for MB-GREEN and 530 nm/590 nm for MB-RED. This fluorescence in wells without template was typically 10,000 to 20,000 fluorescence "units", with about 75% emanating from the fluorometer background and the remainder from the MB probes. The plates were then placed in a thermal cycler for asymmetric amplification at the following temperatures: 94° for one minute; 10 - 15 cycles of 94° for 15 sec, 55° for 15 sec., 70° for 15 seconds; 60° for five minutes. The plates were then incubated at room temperature for at least 20 minutes and fluorescence measured as described above. The fluorescence readings obtained were stable for several hours. Specific fluorescence was defined as the difference in fluorescence before and after the asymmetric amplification. RED/GREEN ratios were defined as the specific fluorescence of MB-RED divided by that of MB-GREEN. RED/GREEN ratios were normalized to the ratio exhibited by the positive controls (25 genome equivalents of DNA from normal cells, as defined in Materials and Methods). We found that the ability of MB probes to discriminate between WT and mutant sequences under our conditions could

not be reliably determined from experiments in which they were tested by hybridization to relatively short complementary single stranded oligonucleotides, and that actual PCR products had to be used for validation.

EXAMPLE 3

Oligonucleotides and DNA sequencing. Primer F1: 5'-CATGTTCTAATATAGTCACATTTTCA-3'; Primer R1: 5'-TCTGAATTAGCTGTATCGTCAAGG-3'; Primer INT: 5'-TAGCTGTATCGTCAAGGCAC-3'; MB-RED: 5'-Cy3-CACGGGCCTGCTGAAAATGACTGCGTG-Dabcyl-3'; MB-GREEN: 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 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'. Sequencing was performed using fluorescently-labeled ABI Big Dye terminators and an ABI 377 automated sequencer.

EXAMPLE 4

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

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

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

Practical Considerations.

Numerous conditions were optimized to define conditions that could be reproducibly and generally applied. As outlined in Fig. 1A, the first step involves amplification from single template molecules. Most protocols for amplification from small numbers of template molecules use a nesting procedure, wherein a product resulting from one set of primers is used as template in a second reaction employing internal primers. As many applications of digital amplification are expected to require hundreds or thousands of separate amplifications, such nesting would be inconvenient and could lead to contamination problems. Hence, conditions were sought that would achieve robust amplification without nesting. The most important of these conditions involved the use of a polymerase that was activated only after heating and optimized concentrations of dNTP's, primers, buffer components, and temperature. The conditions specified in Examples 1-3 were defined after individually optimizing each of these components and proved suitable for amplification of several different human genomic DNA sequences. Though the time required for PCR was not particularly long (~2.5 hr), the number of cycles used was high and excessive compared to the number of cycles required to amplify the "average" single template molecule. The large cycle number was necessary because the template in some wells might not begin to be amplified until several PCR cycles had been completed. The large number of cycles ensured that every well (not simply the average well) would generate a substantial and roughly equal amount of PCR product if a template molecule were present within it.

The second step in Fig 1A involves the detection of these PCR products. It was necessary to considerably modify the standard MB probe approach in order for it to function efficiently in Digital Amplification applications. Theoretically, one separate MB probe could be used to detect

each specific mutation that might occur within the queried sequence. By inclusion of one MB corresponding to WT sequence and another corresponding to mutant sequence, the nature of the PCR product would be revealed. Though this strategy could obviously be used effectively in some situations, it becomes complex when several different mutations are expected to occur within the same queried sequence. For example, in the *c-Ki-Ras* gene example explored here, twelve different base substitutions resulting in missense mutations could theoretically occur within codons 12 and 13, and at least seven of these are observed in naturally-occurring human cancers. To detect all twelve mutations as well as the WT sequence with individual Molecular Beacons would require 13 different probes. Inclusion of such a large number of MB probes would not only raise the background fluorescence but would be expensive. We therefore attempted to develop a single probe that would react with WT sequences better than any mutant sequence within the queried sequence. We found that the length of the loop sequence, its melting temperature, and the length and sequence of the stem were each important in determining the efficacy of such probes. Loops ranging from 14 to 26 bases and stems ranging from 4 to 6 bases, as well as numerous sequence variations of both stems and loops, were tested during the optimization procedure. For discrimination between WT and mutant sequences (MB-GREEN probe), we found that a 16 base pair loop, of melting temperature (Tm) 50-51□, and a 4 bp stem, of sequence 5'-CACG-3', were optimal. For MB-RED probes, the same stem, with a 19-20 bp loop of Tm 54-56□, proved optimal. The differences in the loop sizes and melting temperatures between MB-GREEN and MB-RED probes reflected the fact that only the GREEN probe is designed to discriminate between closely related sequences, with a shorter region of homology facilitating such discrimination.

Examples of the ratios obtained in replicate wells containing DNA templates from colorectal tumor cells with mutations of *c-Ki-Ras* are shown in

Fig. 2. In this experiment, fifty copies of genomic DNA equivalents were diluted into each well prior to amplification. Each of six tested mutants yielded ratios of RED/GREEN fluorescence that were significantly in excess of the ratio obtained with DNA from normal cells (1.5 to 3.4 in the mutants compared to 1.0 in normal DNA; $p < 0.0001$ in each case, Student's t-Test). The reproducibility of the ratios can be observed in this figure. Direct DNA sequencing of the PCR products used for fluorescence analysis showed that the RED/GREEN ratios were dependent on the relative fraction of mutant genes within the template population (Fig. 2). Thus, the DNA from cells containing one mutant *C-Ki-Ras* allele per every two WT *c-Ki-Ras* allele yielded a RED/GREEN ratio of 1.5 (Gly12Arg mutation) while the cells containing three mutant *c-Ki-Ras* alleles per WT allele exhibited a ratio of 3.4 (Gly12Asp). These data suggested that wells containing only mutant alleles (no WT) would yield ratios in excess of 3.0, with the exact value dependent on the specific mutation.

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

EXAMPLE 5

Analysis of DNA from tumor cells. The principles and practical considerations described above was demonstrated with DNA from two colorectal cancer cell lines, one with a mutation in *c-Ki-Ras* codon 12 and the other in codon 13. Representative examples of the MB-RED fluorescence values obtained are shown in Fig. 3. There was a clear biphasic distribution, with "positive" wells yielding values in excess of 10,000 specific fluorescence units (SFU, as defined in Materials and Methods) and "negative" wells yielding values less than 3500 SFU. Gel electrophoreses of 127 such wells demonstrated that all positive wells, but no negative wells, contained PCR products of the expected size (Fig. 3). The RED/GREEN fluorescence ratios of the positive wells are shown in Fig. 4. Again, a biphasic distribution was observed. In the experiment with the tumor containing a Gly12Asp mutation, 64% of the positive wells exhibited RED/GREEN ratios in excess of 3.0 while the other 36% of the positive wells exhibited ratios ranging from 0.8 to 1.1. In the case of the tumor with the Gly13Asp mutation, 54% of the positive wells exhibited RED/GREEN ratios >3.0 while the other positive wells yielded ratios ranging from 0.9 to 1.1. The PCR products from 16 positive wells were used as sequencing templates (Fig. 4). All the wells yielding a ratio in excess of 3.0 were found to contain mutant c-Ki-Ras fragments of the expected sequence, while WT sequence was found in the other PCR products. The presence of homogeneous WT or mutant sequence confirmed that the amplification products were usually derived from single template molecules. The ratios of WT to mutant PCR products determined from the Digital Amplificationassay 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 Amplificationexperiment. As positive controls, 48 of the wells contained 25 genome equivalents of DNA (defined in Materials and Methods) from normal cells. Another 48 wells served as negative controls (no DNA template added). The other 288 wells contained an appropriate dilution of stool DNA. MB-RED fluorescence indicated that 102 of these 288 experimental wells contained PCR products (mean +/- s.d. of 47,000 +/- 18,000 SFU) while the other 186 wells did not (2600 +/- 1500 SFU). The RED/GREEN ratios of the 102 positive wells suggested that five contained mutant c-Ki-Ras genes, with ratios ranging from 2.1 to 5.1. The other 97 wells exhibited ratios ranging from 0.7 to 1.2, identical to those observed in the positive control wells. To determine the nature of the mutant *c-Ki-Ras* genes in the five positive wells from stool, the PCR products were directly sequenced. The four wells exhibiting RED/GREEN ratios in excess of 3.0 were completely composed of mutant c-Ki-Ras sequence (Fig. 5B). The sequence of three of these PCR products revealed Gly12Ala mutations (GGT to GCT at codon 12), while the sequence of the fourth indicated a silent C to T transition at the third position of codon 13. This transition presumably resulted from a PCR error during the first productive cycle of amplification from a WT template. The well with a ratio of 2.1 contained a ~1:1 mix of WT and Gly12Ala mutant sequences. Thus 3.9% (4/102) of the *c-Ki-Ras* alleles present in this stool sample contained a Gly12Ala mutation. The mutant alleles in the stool presumably arose from the colorectal cancer of the patient, as direct sequencing of PCR products generated from DNA of the cancer revealed the identical Gly12Ala mutation (not shown).

CLAIMS

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

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

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

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

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

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

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

4. The method of claim 1 wherein the step of diluting is performed until all of the assay samples yield an amplification product when subjected to a

polymerase chain reaction and each assay sample contains less than 100 nucleic acid template molecules containing a reference genetic sequence.

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

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

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

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

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

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

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

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

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

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

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

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

17. The method of claim 13 wherein the molecular beacon probe detects a wild-type nucleic acid better than a mutant nucleic acid.

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

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

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

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

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

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

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

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

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

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

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

ABSTRACT

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

JOINT DECLARATION FOR PATENT APPLICATION

As the below named inventor, we hereby declare that:

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

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

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

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

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

Prior Foreign Application(s)

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

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

Prior United States Provisional Application(s)

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

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

Prior United States Application(s)

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

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

Power of Attorney

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

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EVANS, Thomas L.	35,805	MEECE, Timothy C.	38,553	VAN ES, J. Pieter	37,746
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FISHER, William J.	32,133	MILLER, Charles L.	43,805	WOLFFE, Franklin D.	19,724
GLEMBOCKI, Christopher	R.38,800	MITRIUS, Janice V.	43,808	WOLFFE, Susan A.	33,568
HANLON, Brian E.	40,449	MORENO, Christopher P.	38,566	WRIGHT, Bradley C.	38,061
HEMMENDINGER, Lisa M.	42,653	NELSON, Jon O.	24,566		
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We hereby declare that all statements made herein of our own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code and that such willful false statements may jeopardize the validity of the application or any patent issuing thereon.

Signature _____ Date 11/28/00

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Fig. 1A

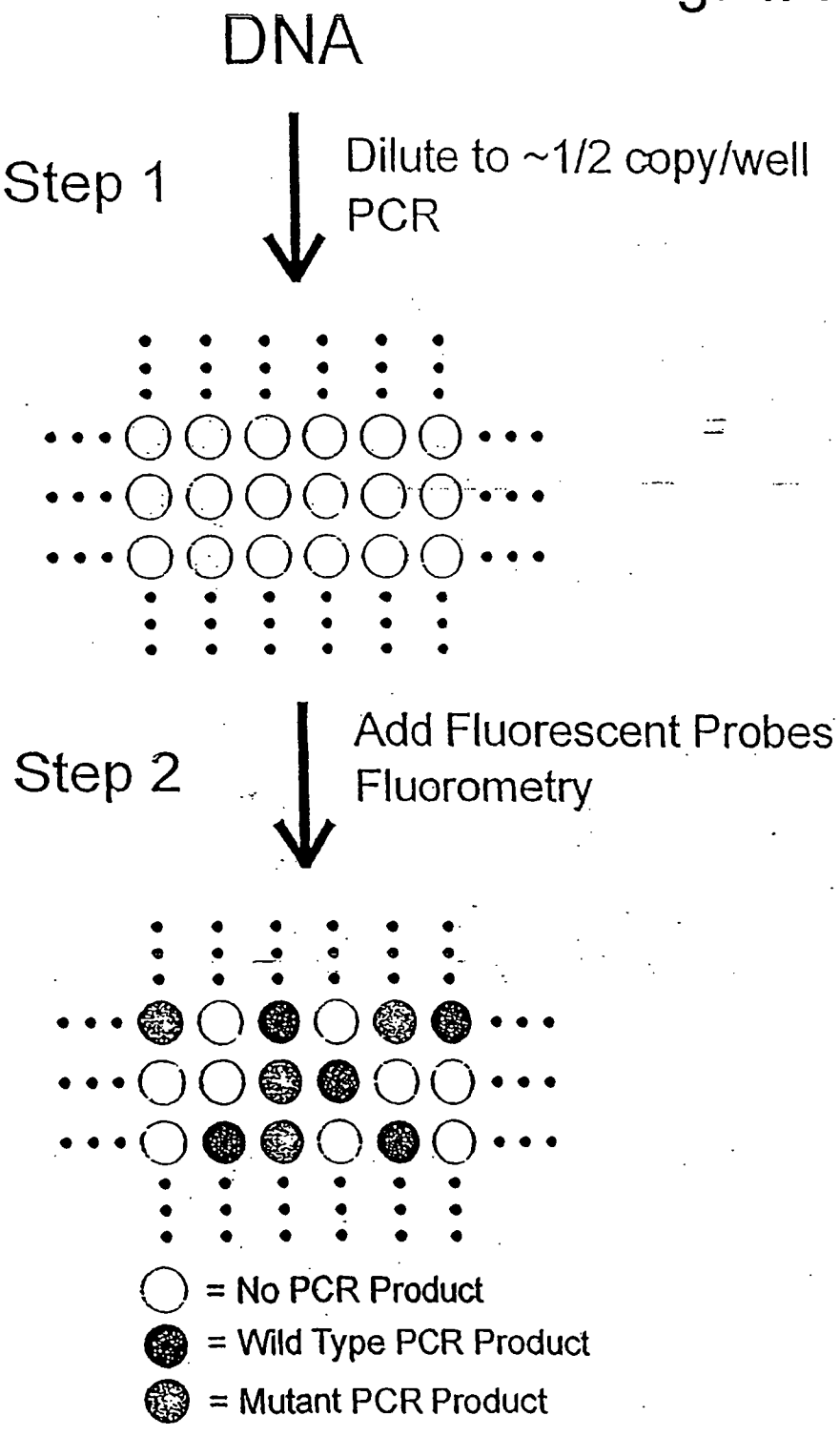

DNA
Step 1
Dilute to ~1/2 copy/well
PCR
Step 2
Add Fluorescent Probes
Fluorometry
= No PCR Product
= Wild Type PCR Product
= Mutant PCR Product

Fig. 1B

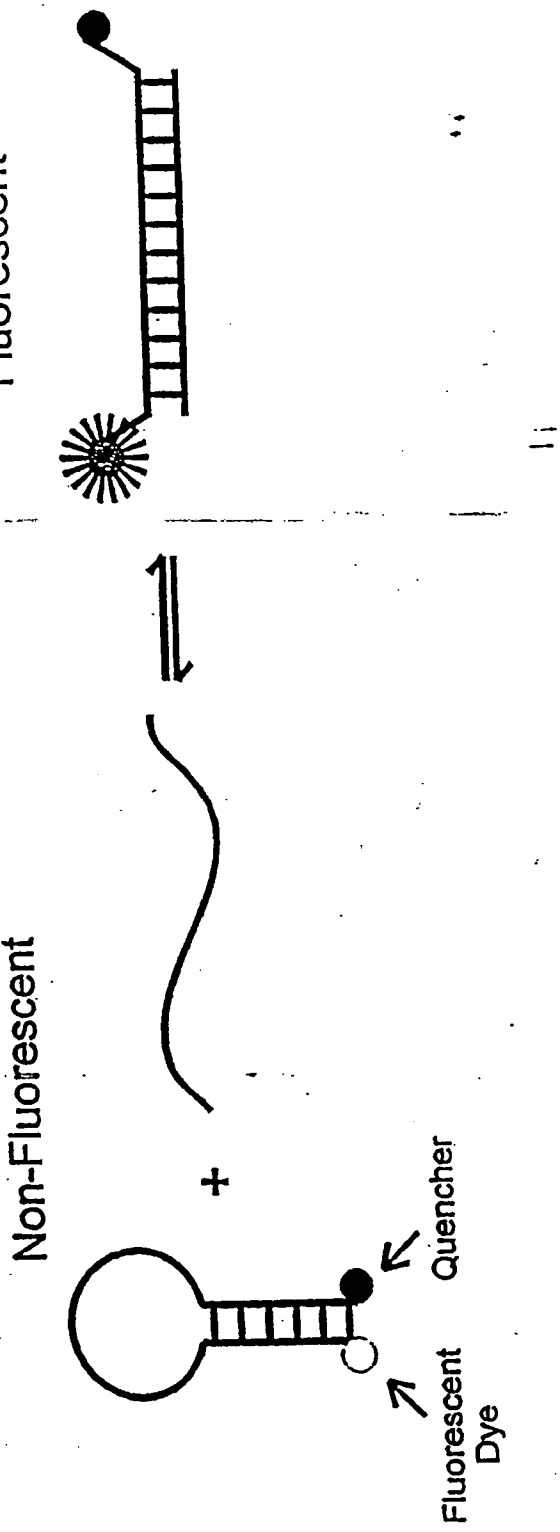


Fig. 1C

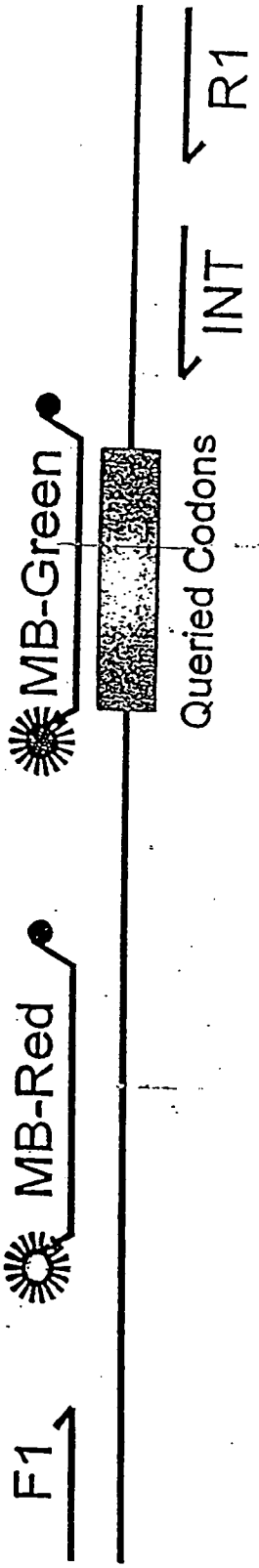
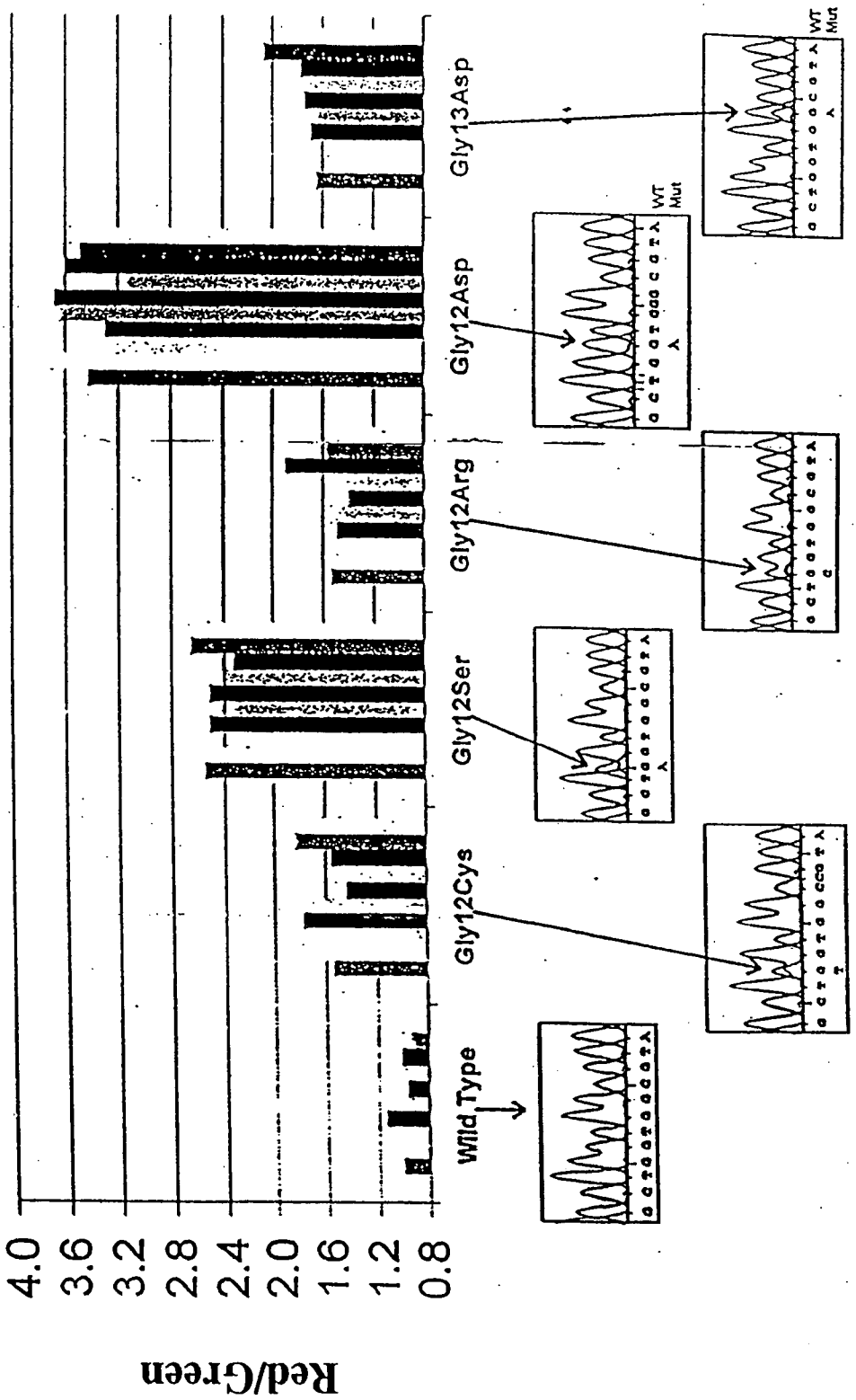


Fig. 2


Red/Green
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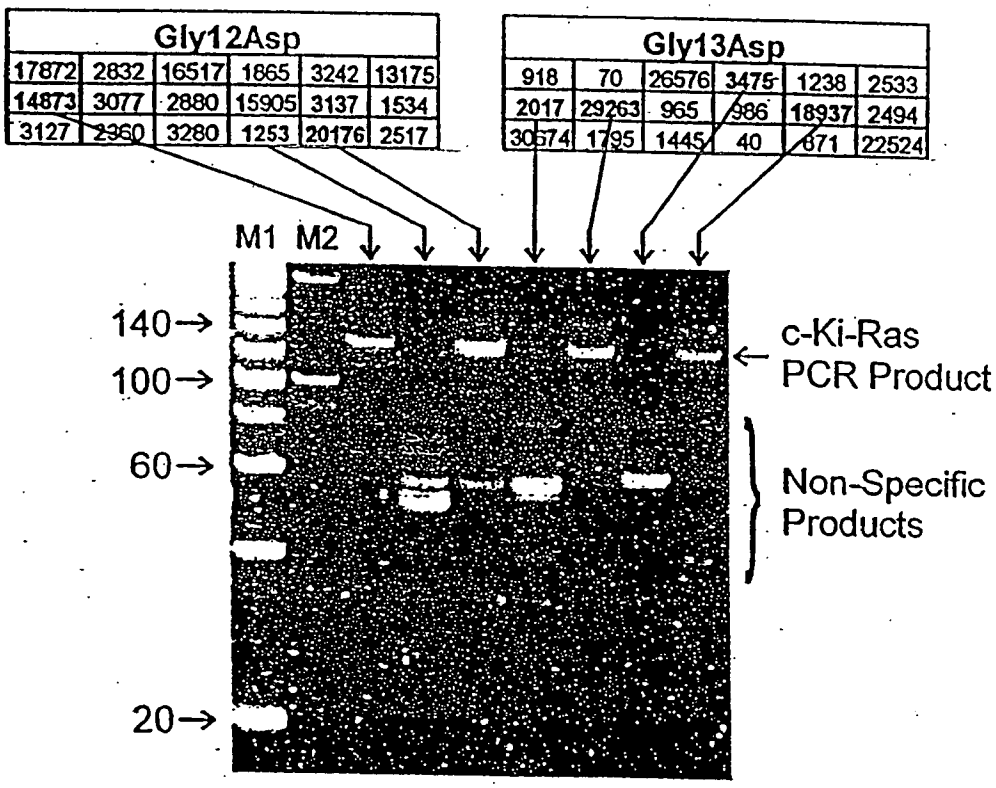
3.6
3.2
2.8
2.4
2.0
1.6
1.2
0.8
Wild Type
Gly12Cys
Gly12Ser
Gly12Arg
Gly12Asp
Gly13Asp
WT
Mut

Fig. 3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3127 2360 3280 1253 20176 2517
Gly13Asp
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2017 29263 965 986 18937 2494
30574 1795 1445 40 671 22524
M1 M2
140→
100→
60→
20→
c-Ki-Ras
PCR Product
Non-Specific
Products

Fig. 4

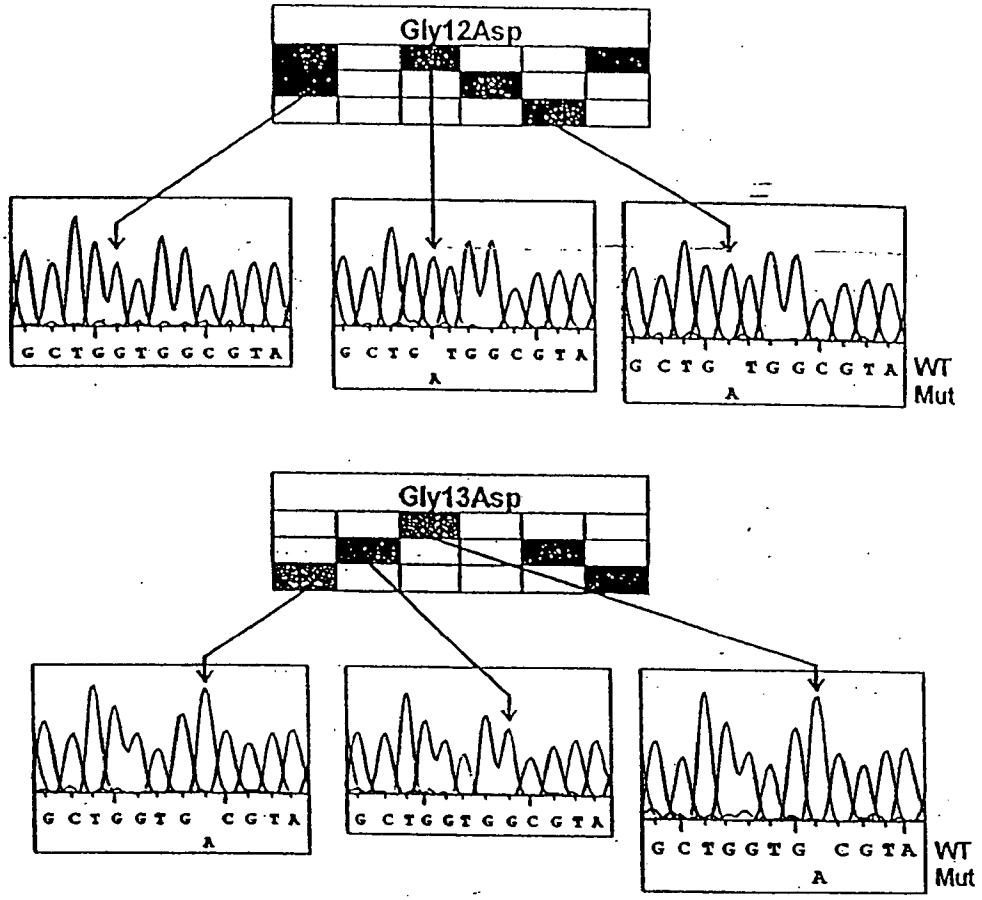
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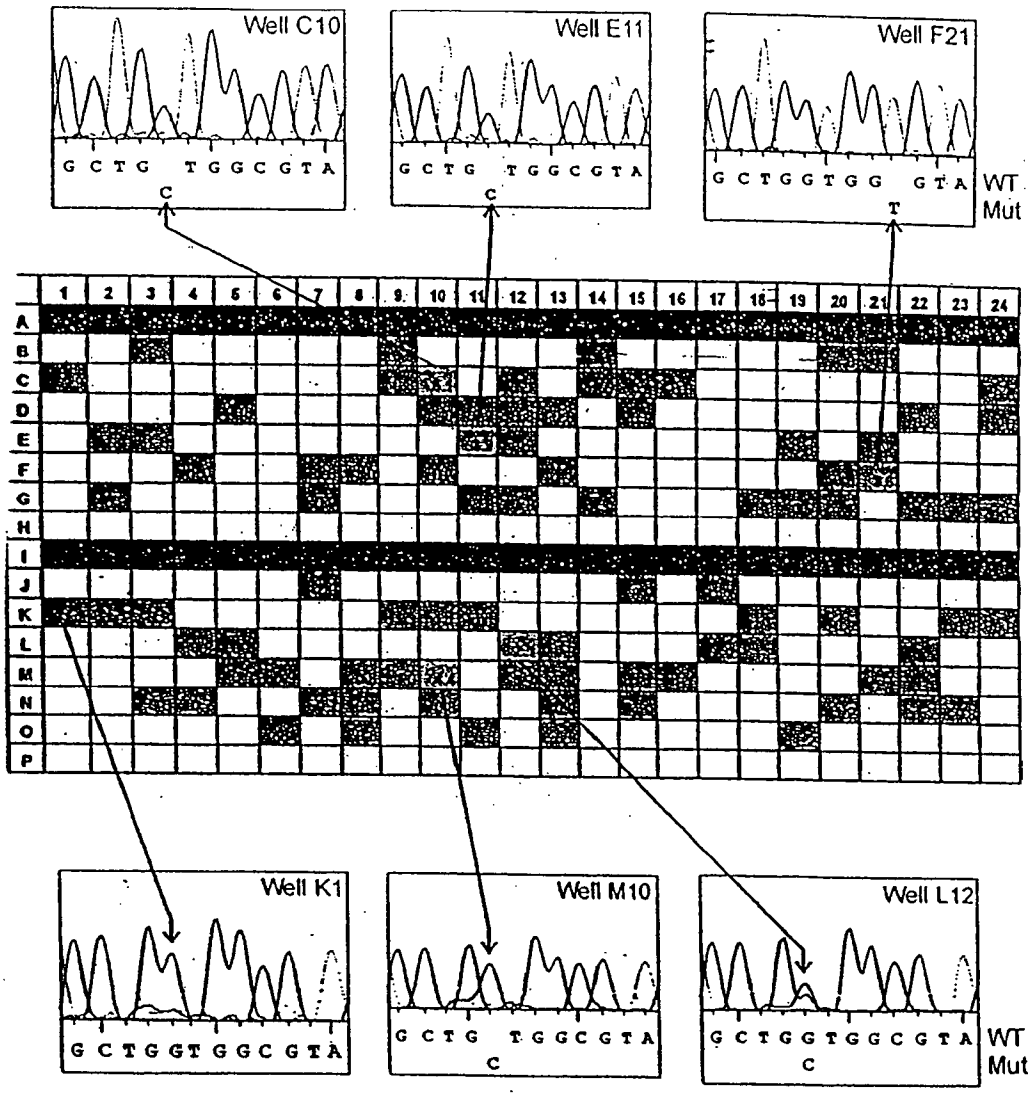
Fig. 5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PTO/SB/06 (12-04)
Approved for use through 7/31/2006. OMB 0651-0032
U.S. Patent and Trademark Office; U.S. DEPARTMENT OF COMMERCE

PATENT APPLICATION FEE DETERMINATION RECORD

Substitute for Form PTO-875 Effective December 8, 2004

Application or Docket Number: 11/097,142

APPLICATION AS FILED – PART I

FOR	(Column 1) NUMBER FILED	(Column 2) NUMBER EXTRA	SMALL ENTITY RATE ($)	SMALL ENTITY FEE ($)	OR	OTHER THAN SMALL ENTITY RATE ($)	OTHER THAN SMALL ENTITY FEE ($)
BASIC FEE (37 CFR 1.16(a), (b), or (c))	N/A	N/A	N/A	150.00		N/A	300.00
SEARCH FEE (37 CFR 1.16(k), (l), or (m))	N/A	N/A	N/A	$250		N/A	$500
EXAMINATION FEE (37 CFR 1.16(o), (p), or (q))	N/A	N/A	N/A	$100		N/A	$200
TOTAL CLAIMS (37 CFR 1.16(i))	28 minus 20 =	8	X$ 25 =	200	OR	X$50 =	
INDEPENDENT CLAIMS (37 CFR 1.16(h))	1 minus 3 =	*	X100 =			X200 =	
APPLICATION SIZE FEE (37 CFR 1.16(s))	If the specification and drawings exceed 100 sheets of paper, the application size fee due is $250 ($125 for small entity) for each additional 50 sheets or fraction thereof. See 35 U.S.C. 41(a)(1)(G) and 37 CFR 1.16(s).						
MULTIPLE DEPENDENT CLAIM PRESENT (37 CFR 1.16(j))			+180=			+360=	
			TOTAL	700		TOTAL	

* If the difference in column 1 is less than zero, enter "0" in column 2.

APPLICATION AS AMENDED – PART II

AMENDMENT A

	(Column 1) CLAIMS REMAINING AFTER AMENDMENT		(Column 2) HIGHEST NUMBER PREVIOUSLY PAID FOR	(Column 3) PRESENT EXTRA	SMALL ENTITY RATE ($)	SMALL ENTITY ADDITIONAL FEE ($)	OR	OTHER THAN SMALL ENTITY RATE ($)	OTHER THAN SMALL ENTITY ADDITIONAL FEE ($)
Total (37 CFR 1.16(i))	*	Minus	**	=	X$ 25 =		OR	X$50 =	
Independent (37 CFR 1.16(h))	*	Minus	***	=	X100 =		OR	X200 =	
Application Size Fee (37 CFR 1.16(s))									
FIRST PRESENTATION OF MULTIPLE DEPENDENT CLAIM (37 CFR 1.16(j))					+180=		OR	+360=	
					TOTAL ADD'L FEE		OR	TOTAL ADD'L FEE	

AMENDMENT B

	(Column 1) CLAIMS REMAINING AFTER AMENDMENT		(Column 2) HIGHEST NUMBER PREVIOUSLY PAID FOR	(Column 3) PRESENT EXTRA	RATE ($)	ADDITIONAL FEE ($)	OR	RATE ($)	ADDITIONAL FEE ($)
Total (37 CFR 1.16(i))	*	Minus	**	=	X$ 25 =		OR	X$50 =	
Independent (37 CFR 1.16(h))	*	Minus	***	=	X100 =		OR	X200 =	
Application Size Fee (37 CFR 1.16(s))									
FIRST PRESENTATION OF MULTIPLE DEPENDENT CLAIM (37 CFR 1.16(j))					+180=		OR	+360=	
					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.

PATENT APPLICATION SERIAL NO. _____

U.S. DEPARTMENT OF COMMERCE
PATENT AND TRADEMARK OFFICE
FEE RECORD SHEET

02/26/2007 SSITHIB1 00000051 190733 11709742

01 FC:2011 150.00 DA
02 FC:2111 250.00 DA
03 FC:2311 100.00 DA
04 FC:2202 200.00 DA

PTO-1556
(5/87)

*U.S. Government Printing Office: 2002 — 466-247/69033

PATENT

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re Application of:	)	
	)	Prior Group Art Unit: 1637
Bert Vogelstein et al.	)	
	)	Prior Examiner: M. Baughman
Serial No.: To Be Assigned	)	
	)	
Filed: February 23, 2007	)	Atty. Dkt. No. 001107.00638

For: DIGITAL AMPLIFICATION

INFORMATION DISCLOSURE STATEMENT

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

Sir:

In accordance with 37 C.F.R. §§ 1.97 and 1.98, enclosed is PTO Form-1449 listing documents for consideration by the Examiner during the prosecution of the subject application. All cited art was previously disclosed or cited in parent application Serial No. 10/828,295 filed April 21, 2004. Copies of the cited art are available in the parent application.

Respectfully submitted,

Date: February 22, 2007

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

Banner & Witcoff, Ltd.
Customer No. 22907

PTO/SB/08A (10-01)
Approved for use through 10/31/2002. OMB 0651-0031
U.S. Patent and Trademark Office: U.S. DEPARTMENT OF COMMERCE

Substitute for form 1449A/PTO

INFORMATION DISCLOSURE STATEMENT BY APPLICANT

(use as many sheets as necessary)

Sheet 1 of 3

Complete if Known	
Application Number	TBA 3
Filing Date	February 22, 2007
First Named Inventor	Bert Vogelstein et al.
Prior Group Art Unit	1637
Prior Examiner Name	M. Baughman
Attorney Docket Number	001107.00638

U.S. PATENT DOCUMENTS

Examiner Initials *	Cite No.[1]	Document Number Number - Kind Code[2] (*if known*)	Publication Date MM-DD-YYYY	Name of Patentee or Applicant of Cited Document	Pages, Columns, Lines, Where Relevant Passages or Relevant Figures Appear
		US-5,213,961	05-25-93	Bunn et al	
		US-5,736,333	04-07-98	Livak et al	
		US-5,518,901	05-21-96	Murtagh	
		US-5,804,383	09-08-1998	Gruenert et al.	
		US- 5,858,663	01-12-1999	Nisson et al.	
		US- 5,670,325	09-1997	Lapidus et al. *	
		US- 6,037,130	03-14-2000	Tyagi et al.	
		US- 5,925,517	07-20-1999	Tyagi et al.	
		US- 5,928,870	07-1999	Lapidus et al. *	
		US- 6,020,137	02-2000	Lapidus et al. *	
		US- 6,143,496	11-2000	Brown et al. *	
		US- 6,291,163	09-18-01	Sidransky	
		US-			
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		US-			

FOREIGN PATENT DOCUMENTS

Examiner Initials*	Cite No.[1]	Foreign Patent Document Country Code[3] - Number[4] - Kind Code[5] (*if known*)	Publication Date MM-DD-YYYY	Name of Patentee or Applicant of Cited Document	Pages, Columns, Lines, Where Relevant Passages or Relevant Figures Appear	T[6]
		WO 95/13399	05-18-1995			
		EP 0643140 A	03-15-1995			
		WO 99/13113	03-18-1999			

Examiner Signature		Date Considered	

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

[1] Applicant's unique citation designation number (optional). [2] See Kinds Codes of USPTO Patent Documents at www.uspto.gov or MPEP 901.04. [3] Enter Office that issued the document, by the two-letter code (WIPO Standard ST.3). [4] For Japanese patent documents, the indication of the year of the reign of the Emperor must precede the serial number of the patent document. [5] Kind of document by the appropriate symbols as indicated on the document under WIPO Standard ST. 16 if possible. [6] 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.

PTO/SB/08B(10-01)
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U.S. Patent and Trademark Office: U.S. DEPARTMENT OF COMMERCE
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INFORMATION DISCLOSURE STATEMENT BY APPLICANT

(use as many sheets as necessary)

Sheet 2 | 3

Complete if Known	
Application Number	TBA
Filing Date	February 22, 2007
First Named Inventor	Bert Vogelstein et al.
Group Art Unit	1637
Examiner Name	M. Baughman
Attorney Docket Number	001107.00638

OTHER PRIOR ART -- NON PATENT LITERATURE DOCUMENTS

Examiner Initials *	Cite No.[1]	Include name of the author (in CAPITAL LETTERS), title of the article (when appropriate), title of the item (book, magazine, journal, serial, symposium, catalog, etc.), date, page(s), volume-issue number(s), publisher, city and/or country where published.	T[2]
		A. PIATEK et al., "Molecular Beacon Sequence Analysis for Detecting Drug Resistance in Mycobacterium Tuberculosis", Nature Biotechnology, April 1998, pp. 359-363, Vol. 16, 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., "Multilex 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	
		B. VOGELSTEIN et al., "Digital PCR", Proceedings of the National Academy of Sciences of the United States, August 3, 1999, pp. 9236-9241, Vol. 96, No. 16	
		K. D.E. EVERETT et al, "Identification of nine species of the Chlamydiaceae Uisng PCR-RFLP", April 1999, pp. 803-813, Vol. 49, No. 2 *	
		Darren G. MONCKTON, *et al.*, "Minisatellite "Isoallele" Discrimination in Pseudohomozygotes by Single Molecule PCR and Variant Repeat Mapping", Genomics 11, pp. 465-467, 1991 *	
		Gualberto RUANO, *et al.*, "Haplotype of Multiple Polymorphisms Resolved by Enzymatic Amplification of Single DNA Molecules", Proc. National Science USA, 1990 *	
		W. NAVIDI, *et al.*, "Using PCR in Preimplantation Genetic Disease Diagnosis", Human Reproduction, Vol. 6, No. 6, pp. 836-849, 1991 *	
		Hongua LI, *et al.*, "Amplification and Analysis of DNA Sequences in Single Human Sperm and Diploid Cells", Nature, Vol. 335, September 29, 1988 *	

Examiner Signature		Date Considered	

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

[1] Unique citation designation number (optional). [2] 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.

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U.S. Patent and Trademark Office: U.S. DEPARTMENT OF COMMERCE
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INFORMATION DISCLOSURE STATEMENT BY APPLICANT

(use as many sheets as necessary)

Sheet 3 | 3

Complete if Known	
Application Number	TBA
Filing Date	February 22, 2007
First Named Inventor	Bert Vogelstein et al.
Group Art Unit	1637
Examiner Name	M. Baughman
Attorney Docket Number	001107.00638

OTHER PRIOR ART -- NON PATENT LITERATURE DOCUMENTS

Examiner Initials *	Cite No.[1]	Include name of the author (in CAPITAL LETTERS), title of the article (when appropriate), title of the item (book, magazine, journal, serial, symposium, catalog, etc.), date, page(s), volume-issue number(s), publisher, city and/or country where published.	T[2]
		Lin ZHANG, *et al.*, "Whole Genome Amplification from a Single Cell: Implications for Genetic Analysis", Proc. National Science USA, Vol. 89, pp. 5847-5851, July 1992 *	
		David SIDRANSKY, *et al.*, "Clonal Expansion of p53 Mutant Cells is Associated with Brain Tumour Progression", Nature, February 27, 1992 *	
		Alec J. JEFFREYS, *et al.*, "Mutation Processes at Human Minisatellites", Electophoresis, 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", Forensic Science International, Vol. 66, pp. 129-141, 1994 *	
		Paul M. LIZARDI, *et al.*, "Mutation Detection and Single-Molecule Counting Using Isothermal Rolling-Circle Amplification", Nature Genetics, Vol. 19, July 1998 *	
		R. PARSONS, *et al.*, "Mismatch Repair Deficiency in Phenotypically Normal Human Cells", Science, Vol. 268, May 5 1995 *	
		MARRAS et al., "Multiplex Detection of Single-Nucleotide Variations Using Molecular Beacons," Genetic Analysis: Biomolecular Engineering, Feb. 1999, 14; 151-156	
		WHITCOMB et al., "Detection of PCR Products Using Self-Probing Amplicons and Fluorescence," Nature Biotechnology, August 1999, Vol. 17, 804-807	

Examiner Signature		Date Considered	

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

[1] Unique citation designation number (optional). [2] 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.

Application Data Sheet

Application Information

Application number::	TBD
Filing Date::	February 23, 2007
Application Type::	Regular
Subject Matter::	Utility
Suggested classification::	
Suggested Group Art Unit::	
CD-ROM or CD-R?::	None
Number of CD disks::	
Number of copies of CDs::	
Sequence submission?::	Paper
Computer Readable Form (CRF)?::	NO
Number of copies of CRF::	
Title::	DIGITAL AMPLIFICATION
Attorney Docket Number::	001107.00638
Request for Early Publication?::	NO
Request for Non-Publication?::	NO
Suggested Drawing Figure::	
Total Drawing Sheets::	7
Small Entity?::	YES
Latin name::	
Variety denomination name::	
Petition included?::	NO
Petition Type::	
Licensed US Govt. Agency::	National Institutes of Health
Contract or Grant Numbers::	CA 43460, CA 57345 & CA 62924
Secrecy Order in Parent Appl.?::	NO

Applicant Information

Applicant Authority Type::	Inventor
Primary Citizenship Country::	US
Status::	Full Capacity
Given Name::	Bert
Middle Name::	
Family Name::	Vogelstein
Name Suffix::	
City of Residence::	Baltimore
State or Province of Residence::	MD
Country of Residence::	
Street of mailing address::	3700 Breton Way
City of mailing address::	Baltimore
State or Province of mailing address::	MD
Country of mailing address::	
Postal or Zip Code of mailing address::	21208

Applicant Authority Type::	Inventor
Primary Citizenship Country::	US
Status::	Full Capacity
Given Name::	Kenneth
Middle Name::	
Family Name::	Kinzler
Name Suffix::	
City of Residence::	BelAir
State or Province of Residence::	MD
Country of Residence::	
Street of mailing address::	1403 Halkirk Way
City of mailing address::	BelAir

State or Province of mailing address:: MD

Country of mailing address::

Postal or Zip Code of mailing address:: 21015

Applicant Authority Type:: Inventor

Primary Citizenship Country::

Status:: Full Capacity

Given Name::

Middle Name::

Family Name::

Name Suffix::

City of Residence::

State or Province of Residence::

Country of Residence::

Street of mailing address::

City of mailing address::

State or Province of mailing address::

Country of mailing address::

Postal or Zip Code of mailing address::

Correspondence Information

Correspondence Customer Number:: 22907

Representative Information

Representative Customer Number:: 22907

Domestic Priority Information

Application::	Continuity Type::	Parent Application::	Parent Filing Date::
This Application	Continuation of	10/828,295	04/21/2004

10/828,295	Division of	09/981,356	10/12/01
09/981,356	Continuation of	09/613,826	07/11/00
09/613,826	Non-Provisional of	60/146,792	08/02/99

Foreign Priority Information

Country::	Application number::	Filing Date::	Priority Claimed::

Assignee Information

Assignee name:: The Johns Hopkins University

Street of mailing address:: 3400 N. Charles St.

City of mailing address:: Baltimore

State or Province of mailing address:: MD

Country of mailing address::

Postal or Zip Code of mailing address:: 21218

PATENT

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re Application of	)	Prior Group Art Unit: 1637
	)	
Bert VOGELSTEIN et al	)	Prior Examiner: M. Baughman
	)	
Serial No. TBA	)	
	)	
Filed: February 23, 2007	)	Atty. Dkt. No. 001107.00638

For: DIGITAL AMPLIFICATION

TRANSMITTAL OF SEQUENCE LISTING

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

Sir:

Applicants respectfully request that the Patent Office use the computer readable form of the sequence listing submitted on November 14, 2003 in parent Application Serial Number 09/981,356 for examination of the instant application. I believe the contents of the referenced computer readable form and the paper copy of the sequence listing submitted herewith are identical.

Respectfully submitted,

Date: February 22, 2007

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

Banner & Witcoff, Ltd.
Customer No. 22907

SEQUENCE LISTING

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Kinzler, Kenneth W.

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PTO/SB/06 (12-04)
Approved for use through 7/31/2006. OMB 0651-0032
U.S. Patent and Trademark Office; U.S. DEPARTMENT OF COMMERCE

2/23/2007

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PATENT APPLICATION FEE DETERMINATION RECORD

Substitute for Form PTO-875

Application or Docket Number: 11/709,742

APPLICATION AS FILED – PART I

FOR	(Column 1) NUMBER FILED	(Column 2) NUMBER EXTRA	SMALL ENTITY RATE ($)	SMALL ENTITY FEE ($)	OR	OTHER THAN SMALL ENTITY RATE ($)	OTHER THAN SMALL ENTITY FEE ($)
BASIC FEE (37 CFR 1.16(a), (b), or (c))				150			300
SEARCH FEE (37 CFR 1.16(k), (i), or (m))				250			500
EXAMINATION FEE (37 CFR 1.16(o), (p), or (q))				100			200
TOTAL CLAIMS (37 CFR 1.16(i))	48 minus 20 =	28	X$ 25	700	OR	X$50	
INDEPENDENT CLAIMS (37 CFR 1.16(h))	5 minus 3 =	* 2	105	210		210	
APPLICATION SIZE FEE (37 CFR 1.16(s))	If the specification and drawings exceed 100 sheets of paper, the application size fee due is $250 ($125 for small entity) for each additional 50 sheets or fraction thereof. See 35 U.S.C. 41(a)(1)(G) and 37 CFR						
MULTIPLE DEPENDENT CLAIM PRESENT (37 CFR 1.16(j))						360	
			TOTAL	1410		TOTAL	

* If the difference in column 1 is less than zero, enter "0" in column 2.

APPLICATION AS AMENDED – PART II

AMENDMENT A

	(Column 1) CLAIMS REMAINING AFTER AMENDMENT		(Column 2) HIGHEST NUMBER PREVIOUSLY PAID FOR	(Column 3) PRESENT EXTRA	SMALL ENTITY RATE ($)	SMALL ENTITY ADDI-TIONAL FEE ($)	OR	OTHER THAN SMALL ENTITY RATE ($)	OTHER THAN SMALL ENTITY ADDI-TIONAL FEE ($)
Total (37 CFR 1.16(i))	*	Minus	**	=	X =		OR	X =	
Independent (37 CFR 1.16(h))	*	Minus	***	=	X =		OR	X =	
Application Size Fee (37 CFR 1.16(s))									
FIRST PRESENTATION OF MULTIPLE DEPENDENT CLAIM (37 CFR 1.16(j))							OR	360	
					TOTAL ADD'T FEE*		OR	TOTAL ADD'T FEE	

AMENDMENT B

	(Column 1) CLAIMS REMAINING AFTER AMENDMENT		(Column 2) HIGHEST NUMBER PREVIOUSLY PAID FOR	(Column 3) PRESENT EXTRA	RATE ($)	ADDI-TIONAL FEE ($)	OR	RATE ($)	ADDI-TIONAL FEE ($)
Total (37 CFR 1.16(i))	*	Minus	**	=	X =		OR	X =	
Independent (37 CFR 1.16(h))	*	Minus	***	=	X =		OR	X =	
Application Size Fee (37 CFR 1.16(s))									
FIRST PRESENTATION OF MULTIPLE DEPENDENT CLAIM (37 CFR 1.16(j))					N/A		OR	N/A	
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* 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.

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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EXHIBIT 4

ISSUE CLASSIFICATION	
435	Class
91.2	Subclass

PATENT NUMBER
6440705
6440705

U.S. UTILITY Patent Application

O.I.P.E. SCANNED	PATENT DATE
KW GW QA CL	AUG 27 2002

APPLICATION NO.	CONT/PRIOR	CLASS	SUBCLASS	ART UNIT	EXAMINER
09/613826	D	435	91.2	~~1638~~ 1657	Siew 74907

APPLICANTS: Bert Vogelstein
Kenneth Kinzler

TITLE: Digital amplification

PTO-2040
12/99

ISSUING CLASSIFICATION

ORIGINAL CLASS	ORIGINAL SUBCLASS	CROSS REFERENCE(S) CLASS	CROSS REFERENCE(S) SUBCLASS (ONE SUBCLASS PER BLOCK)					
435	91.2	435	6	7.1	91.1			
INTERNATIONAL CLASSIFICATION		536	22.1	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								

☐ Continued on Issue Slip Inside File Jacket

☐ TERMINAL DISCLAIMER

☐ The term of this patent subsequent to _____ (date) has been disclaimed.

☐ The term of this patent shall not extend beyond the expiration date of U.S Patent. No. _____

☐ The terminal ___ months of this patent have been disclaimed.

DRAWINGS			CLAIMS ALLOWED	
Sheets Drwg.	Figs. Drwg.	Print Fig.	Total Claims	Print Claim for O.G.
7	7	None	64	1

NOTICE OF ALLOWANCE MAILED

_____ (Assistant Examiner) _____ (Date)

March 26, 2002

ISSUE FEE (W)

Jeffrey Siew 3/19/02 (Primary Examiner) (Date)

Amount Due	Date Paid
$1480.00	5-21-02

MONICA R. GRAVES PATENT ANALYST (Acting Primary Examiner) 3/22/02 (Date)

ISSUE BATCH NUMBER
9893

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ISSUE FEE IN FILE

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Formal Drawings ( ) sht(s) rec'd (FACE)

PATENT APPLICATION

09613826

JC869 U.S. PTO
09/613826
07/11/00

Briefed in HCO

INITIALS

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3.	PE C	03-22-01	44.		
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8.	Amdt A Replacing Draft	7/12/01	49.		
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Class	Sub.	Date	Exmr.
435	6	4/7/01	JS
	7.1		
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536	22.1		
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	24.3		
	24.31		
	24.32		
	24.33		
updated		9/20/01	JS
updated		3/19/02	JS

INTERFERENCE SEARCHED

Class	Sub.	Date	Exmr.
435	6	3/19/02	JS
	7.1		
	91.1		
	91.2		
536	22.1		
	23.1		
	24.3-.33		

SEARCH NOTES
(INCLUDING SEARCH STRATEGY)

EAST AIPB STN CAPLUS, MEDLINE, CANCERLIT, BIOSIS SMD reference target single molecule genomic equivalent molecular beacon loop hairpin stem Tm melting temperature	Date	Exmr.
	4/7/01	JS
updated	9/20/01	JS
updated	3/19/02	JS

(RIGHT OUTSIDE)

ISSUE SLIP STAPLE AREA (for additional cross references)

POSITION	INITIALS	ID NO.	DATE
FEE DETERMINATION	[illegible]	6550	7/20/01
O.I.P.E. CLASSIFIER	PH		9/22
FORMALITY REVIEW	AS	551	8-29-01
RESPONSE FORMALITY REVIEW	AS	573	03-22-01

INDEX OF CLAIMS

✓ ..... Rejected
= ..... Allowed
— (Through numeral)... Canceled
÷ ..... Restricted
N ..... Non-elected
I ..... Interference
A ..... Appeal
O ..... Objected

Claim Final	Claim Original	Date
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CONFIRMATION NO. 9893

Bib Data Sheet

SERIAL NUMBER	FILING DATE	CLASS	GROUP ART UNIT	ATTORNEY DOCKET NO.
09/613,826	07/11/2000 RULE	435	1632	01107.00031

APPLICANTS

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

** CONTINUING DATA **********

THIS APPLN CLAIMS BENEFIT OF 60/146,792 08/02/1999

** FOREIGN APPLICATIONS **********

IF REQUIRED, FOREIGN FILING LICENSE GRANTED ** 08/29/2000

** SMALL ENTITY **

Foreign Priority claimed	35 USC 119 (a-d) conditions met	Verified and Acknowledged	STATE OR COUNTRY	SHEETS DRAWING	TOTAL CLAIMS	INDEPENDENT CLAIMS
☐ yes ☑ no	☐ yes ☐ no ☐ Met after Allowance	Examiner's Signature Initials	MD	7	64	5

ADDRESS

22907

TITLE

Digital amplification

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BIBDATASHEET

Bib Data Sheet

CONFIRMATION NO. 9693

SERIAL NUMBER	FILING DATE	CLASS	GROUP ART UNIT	ATTORNEY DOCKET NO.
09/613,826	07/11/2000 RULE	435	1637	01107.00031

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 **********

IF REQUIRED, FOREIGN FILING LICENSE GRANTED ** SMALL ENTITY **
** 08/29/2000

		STATE OR	SHEETS	TOTAL	INDEPENDENT
Foreign Priority claimed	☐ yes ☐ no	COUNTRY	DRAWING	CLAIMS	CLAIMS
35 USC 119 (a-d) conditions met	☐ yes ☐ no ☐ Met after Allowance	MD	7	64	5
Verified and Acknowledged	Examiner's Signature Initials				

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

TITLE
Digital amplification

FILING FEE	FEES: Authority has been given in Paper	☐ All Fees
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DIGITAL AMPLIFICATION

ABSTRACT

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

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).

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

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 ~50 genome equivalents of 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).

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.

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.) 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 amplifable 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.

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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	ccomon 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

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, NY). The composition of the reactions was: 67 mM Tris, pH 8.8, 16.6 mM NH_4SO_4, 6.7 mM $MgCl_2$, 10 mM β-mercaptoethanol, 1 mM dATP, 1 mM dCTP, 1 mM dGTP, 1 mM TTP, 6% DMSO, 1 uM primer F1, 1 uM primer R1, 0.05 units/ul Platinum Taq polymerase (Life Technologies, Inc.), and "one-half genome equivalent" of DNA. To determine the amount of DNA corresponding to one-half genome equivalent, DNA samples were serially diluted and tested via PCR. The amount that yielded amplification products in half the wells, usually ~1.5 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_4SO_4, 6.7 mM $MgCl_2$, 10 mM β-mercaptoethanol, 1 mM dATP, 1 mM dCTP, 1 mM dGTP, 1 mM TTP, 6% DMSO, 5 uM primer INT, 1 uM MB-GREEN, 1 uM MB-RED, 0.1 units/ul Platinum Taq polymerase. The plates were centrifuged for 20 seconds at 6000 g and fluorescence read at excitation/emission wavelengths of 485 nm/530 nm for MB-GREEN and 530 nm/590 nm for MB-RED. 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'; Primer R1: 5'-TCTGAATTAGCTGTATCGTCAAGG-3'; Primer INT:

5'-TAGCTGTATCGTCAAGGCAC-3'; MB-RED: 5'-Cy3-CACGGGCCTGCTGAAAATGACTGCGTG-Dabcyl-3'; MB-GREEN: 5'-Fluorescein-CACGGGAGCTGGTGGCGTAGCGTG-Dabcyl-3'. 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'. 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 6th 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 (Tm) 50-51°, and a 4 bp stem, of sequence 5'-CACG-3', were optimal. For MB-RED probes, the same stem, with a 19-20 bp loop of Tm 54-56°, proved optimal. The differences in the loop sizes and melting temperatures between MB-GREEN and MB-RED probes reflected the fact that only the GREEN probe is designed to discriminate between closely related sequences, with a shorter region of homology facilitating such discrimination.

Examples of the ratios obtained in replicate wells containing DNA templates from colorectal tumor cells with mutations of *c-Ki-Ras* are shown in Fig. 2. In this experiment, fifty 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 illlustrated 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 +/- 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. 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).

References

1. Vogelstein & Kinzler, (1998) The Genetic Basis of Human Cancer (MCGraw-Hill, Toronto).

2. Lee et al., (1997) Free Radical Biol Med 22, 1259-69.

3. Ozawa, T. (1997) Physiol Rev 77, 425-64.

4. Sidransky et al., (1991) Science 252, 706-09.

5. Sidransky et al., (1992) Science 256, 102-05.

6. Smith-Ravin et al., (1995) Gut 36, 81-6.

7. Mills et al., (1995) J. Natl Cancer Inst 87, 1056-60.

8. Mao et al. (1994) Cancer Res 54, 1634-37.

9. Brossart et al., (1995) Caner Res 55, 4065-68.

10. Tada et al. (1993) Cancer Res 53, 2472-74.

11. Nawroz et al. (1996) Nature Med 2, 1035-37.

12. Hayashi et al., (1994) Cancer Res 54, 3853-56.

13. Sidransky, (1997) Science 278, 1054-59.

14. Koch et al. (1994) Arch Otolaryngol Head Neck Surg 120, 943-47.

15. Kumar et al., (1990) Science 248, 1101-04.

16. Jonason et al., (1996) Proc Natl Acad Sci USA 93, 14025-29.

17. Ananthaswamy et al. (1999) J Invest Dermatol 112, 763-68.

18. Bar-Eli et al. (1989) Blood 73, 281-83.

19. Collins et al. (1989) Blood 73, 1028-32.

20. Saiki et al. (1986) Nature 324, 163-66.

21. Bos et al. (1987) Nature 327, 293-97.

22. Dicker et al. (1990) Genes Chromosomes Cancer 1, 257-69.

23. Haque et al. (1998) Diagn Mol Pathol 7, 248-52.

24. Haliassos et al. (1989) Nucleic Acids Res 17, 8093-99.

25. Chen et al. (1997) Anal Biochem 244, 191-94.

26. Cha et al. (1992) PCR Methods Appl 2, 14-20.

27. Jiang et al. (1989) Oncogene 4, 923-28.

28. Kahn et al. (1991) Oncogene 6, 1079-83.

29. Kahn et al. (1995) Methods Enzymol 255, 452-64.

30. Laken et al., (1998) Nature Biotechnol 16, 1352-56.

31. Whitcombe et al. (1998) Curr Opin Biotechnol 9, 602-08.

32. Day et al. (1999) Nucleic Acids Res 27, 1820-18.

33. Tyagi et al. (1998) Nature Biotechnol 16, 49-53.

34. Tyagi et al. (1996) Nat Biotechnol 14, 303-08.

35. Lee et al. (1993) Nucleic Acids Res 21, 3761-66.

36. Chiang et al. (1996) Genome Res 6, 1013-26.

37. Heid et al. (1996) Genome Res 6, 986-94.

38. Paris et al. (1998) Nucleic Acids Res 26, 3789-93.

39. Gibson et al. (1997) Clin Chem 43, 1336-41.

40. Chen et al. (1999) Genome Res 9, 492-98.

41. Szollosi et al. (1998) Cytometry 34, 159-79.

42. Cortopassi et al. (1992) Mutat Res 277, 239-49.

43. Monckton et al. (1991) Genomics 11, 465-67.

44. Chou et al. (1992) Nucleic Acids Res 20, 1717-23.

45. Kellogg et al. (1994) Biotechniques 16, 1134-37.

46. Li et al. (1988) Nature 335, 414-17.

47. Schmitt et al. (1994) Forensic Sci Int 66, 129-41.

48. Navidi et al. (1991) Hum Reprod 6, 836-49.

49. Zhang et al. (1992) Proc Natl Acad Sci USA 89, 5847-51.

50. Jeffreys et al. (1995) Electrophoresis 16, 1577-85.

51. Ruano et al. (1990) Proc Natl Acad Sci USA, 6296-300.

52. Sidransky et al. (1992) Nature 355, 846-47.

53. Parsons et al. (1995) Science 268, 738-40.

54. Lizardi et al. (1998) Nature Genet 19, 225-32.

55. Vogelstein (1989) Science 244, 207-11.

56. Marras et al. (1999) Genet Anal 14, 151-56.

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 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.

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:

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.

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.

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.

add a'

36

PRINT OF DRAWINGS
AS ORIGINALLY FILED

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Fig. 1A

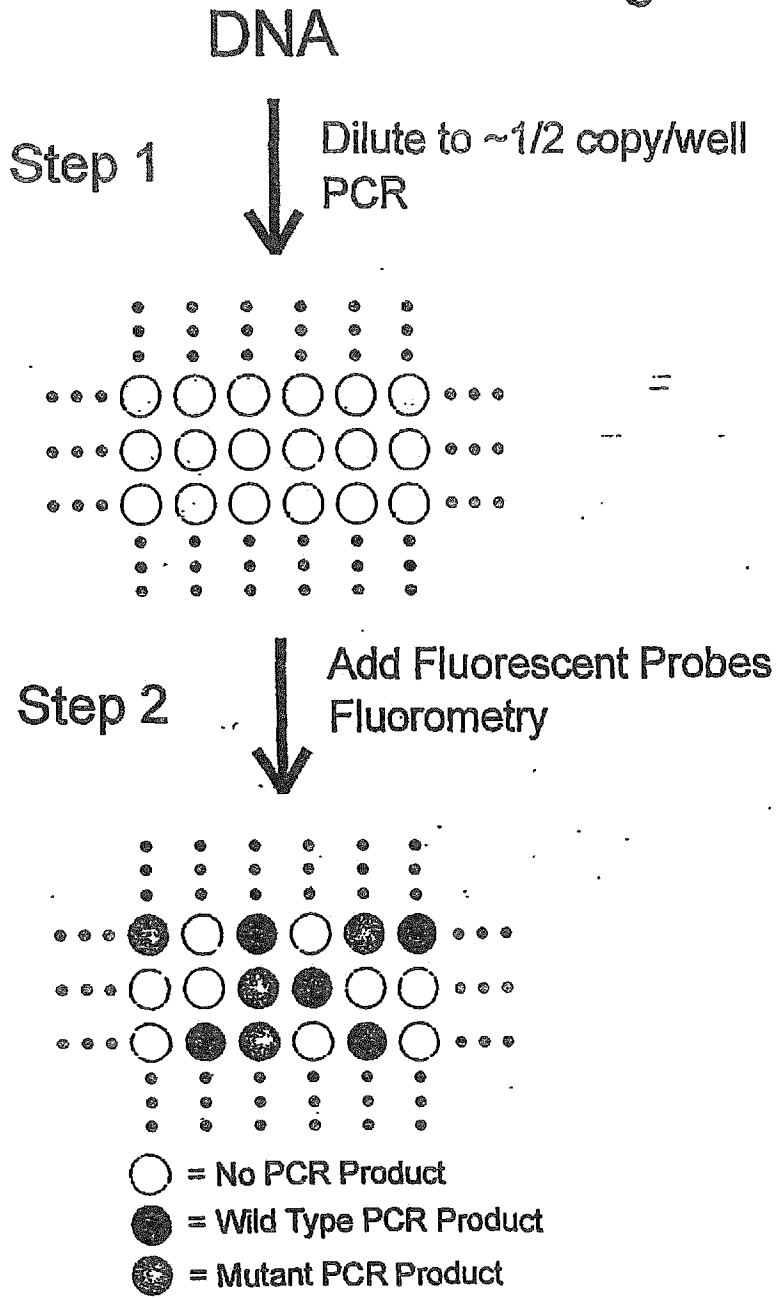

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Fig. 1B

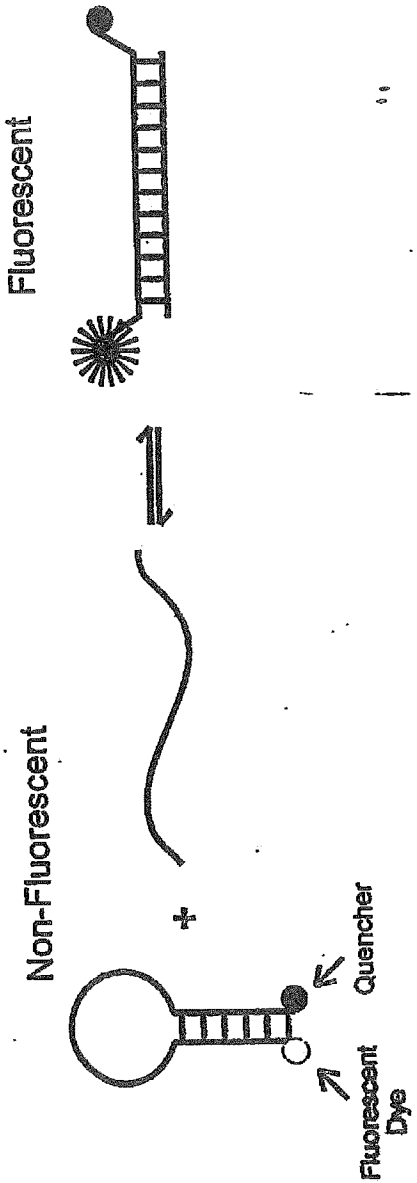

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Fig. 1C

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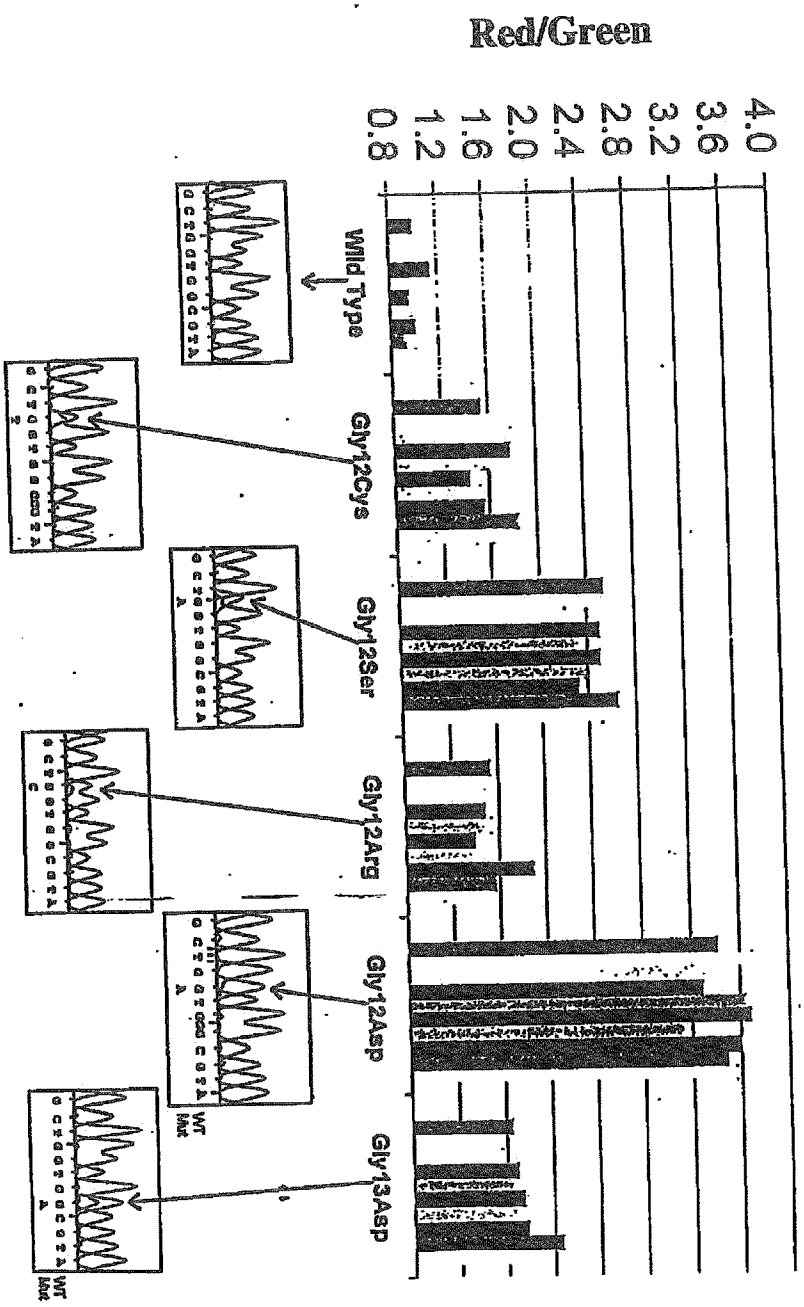

Fig. 2

PRINT OF DRAWINGS
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Fig. 3

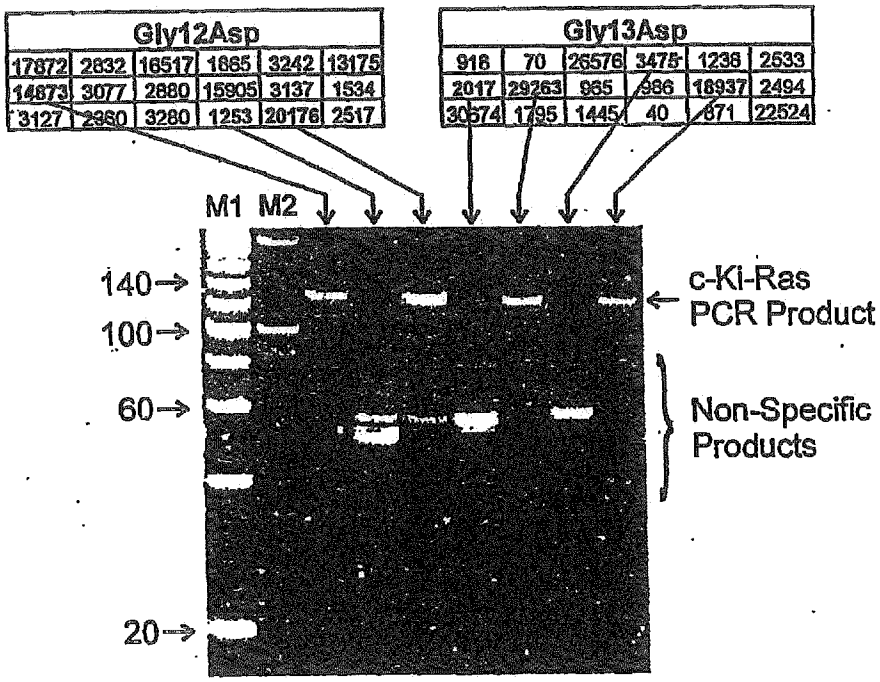

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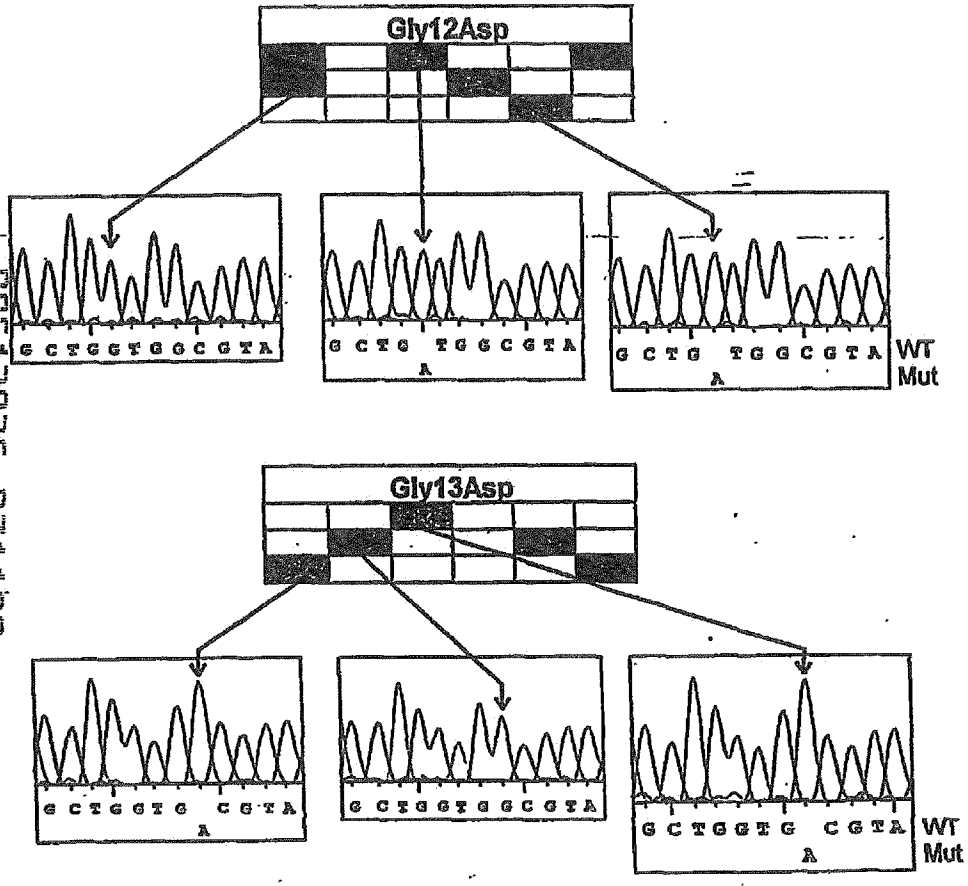
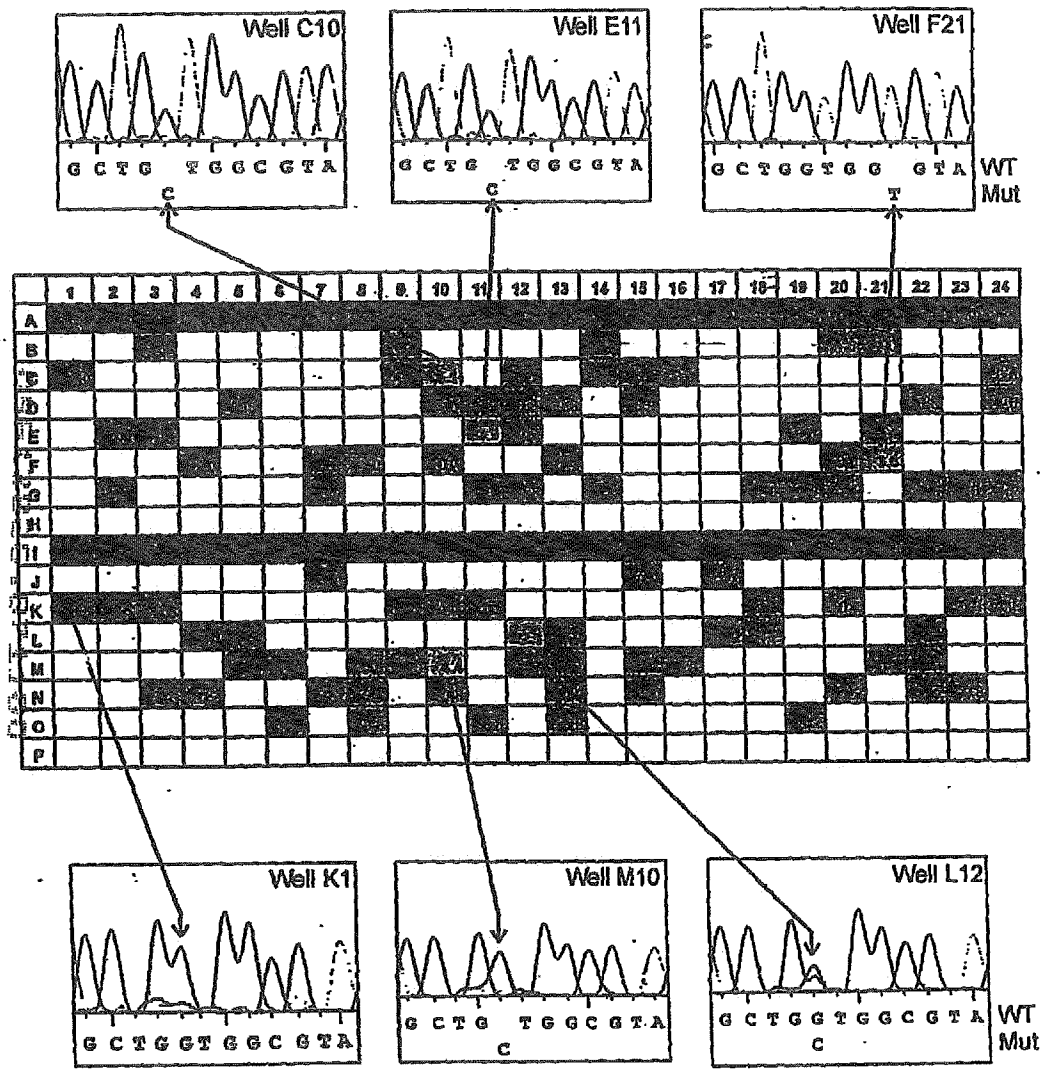
Fig. 4



PRINT OF DRAWINGS
AS ORIGINALLY FILED

Fig. 5



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

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):

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)

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
TOTAL FILING FEE DUE			$819.00
Assignment Recordation Fee (if applicable) (37 C.F.R. § 1.21(h))	0	40.00	$0.00
GRAND TOTAL DUE			$819.00

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

#2

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

OC000000005521419

Date Mailed: 11/01/2000

NOTICE TO FILE MISSING PARTS OF NONPROVISIONAL APPLICATION

FILED UNDER 37 CFR 1.53(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.
  - $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 1.16(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.

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)
)
Filed: July 11, 2000) Docket No. 01107.00031

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
TOTAL FILING FEE	$896.00

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

LARATION FOR PATENT APP. CATION

d inventor, we hereby declare that:

Our resid[illegible]t office address and citizenship are as stated below next to our names;

We believe w[illegible]e the original, first and joint inventors of the subject matter which is claimed and for which a patent is sought on the [illegible]vention entitled DIGITAL AMPLIFICATION, the specification of which

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

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

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

Prior Foreign Application(s)

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

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

Prior United States Provisional Application(s)

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

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

Prior United States Application(s)

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

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

Power of Attorney

this applica
registration numbers

jointly and severally, as our attorneys with full power of substitution and revocation, to prosecute
ss in the Patent and Trademark Office connected herewith the following attorneys and agents, their
heir names:

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

All correspondence and telephone communications should be addressed to:

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

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

We hereby declare that all statements made herein of our own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code and that such willful false statements may jeopardize the validity of the application or any patent issuing thereon.

Signature _____ Date 11/28/00

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

Residence Baltimore, Maryland
Citizenship United States
Post Office Address 3700 Breton Way, Baltimore, Maryland 21208

Signature _____ Date 11/28/00

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

Residence BelAir, Maryland
Citizenship United States
Post Office Address 1403 Halkirk Way, BelAir, Maryland 21015

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

OC000000005521418

Date Mailed: 11/01/2000

NOTICE TO FILE MISSING PARTS OF NONPROVISIONAL APPLICATION

FILED UNDER 37 CFR 1.53(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.
  - $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 1.16(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.**

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12/19/2000 SMUMS
01 FC:201 355.00 CH
02 FC:202 90.00 CH
03 FC:203 312.00 CH
04 FC:205 39.00 CH

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

Customer Service Center
Initial Patent Examination Division (703) 308-1202
PART 2 - COPY TO BE RETURNED WITH RESPONSE

#4/5

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

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
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) 1of 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:_____

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

OTPE DEC 1 5 2000 PATENT & TRADEMARK OFFICE

U.S. PATENT DOCUMENTS

EXAMINER INITIAL	DOCUMENT NUMBER	DATE	NAME	CLASS	SUB CLASS	FILING DATE

FOREIGN PATENT DOCUMENTS

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

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

JS	~~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~~
JS	~~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 Jeffrey Siew	DATE CONSIDERED 4/5/01

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.

#13(S)
4-5-01

PATENT APPLICATION

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re application of:)
Bert Vogelstein, *et al.*) 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

#5

Sheet 1 of 1

PTO-1449 (Modified) U.S. DEPARTMENT OF COMMERCE PATENT AND TRADEMARK OFFICE INFORMATION DISCLOSURE STATEMENT BY APPLICANT	ATTY. DOCKET NO. 01107.00031	SERIAL NUMBER 09/613,926 ~~TBA~~
	APPLICANTS Bert Vogelstein, *et al.*	
	FILING DATE July 11, 2000	GROUP ART UNIT ~~TBA~~ 1637

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MAR 08 2001
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OIPE
MAR 07 2001
PATENT & TRADEMARK OFFICE

U.S. PATENT DOCUMENTS

EXAMINER INITIAL	DOCUMENT NUMBER	DATE	NAME	CLASS	SUB CLASS	FILING DATE
JS	5,670,325	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	Darren G. MONCKTON, *et al.*, "Minisatellite "Isoallele" Discrimination in Pseudohomozygotes by Single Molecule PCR and Variant Repeat Mapping", Genomics 11, pp. 465-467, 1991
JS	Gualberto RUANO, *et al.*, "Haplotype of Multiple Polymorphisms Resolved by Enzymatic Amplification of Single DNA Molecules", Proc. National Science USA, 1990 Vol. 87 pp 6296-6300
JS	W. NAVIDI, *et al.*, "Using PCR in Preimplantation Genetic Disease Diagnosis", Human Reproduction, Vol. 6, No. 6, pp. 836-849, 1991
JS	Hongua LI, *et al.*, "Amplification and Analysis of DNA Sequences in Single Human Sperm and Diploid Cells", Nature, Vol. 335, September 29, 1988 pp 414-417
JS	Ramon PARSONS, *et al.*, "Mismatch Repair Deficiency in Phenotypically Normal Human Cells", Science, Vol. 268, May 5, 1995 pp 738-740
JS	Lin ZHANG, *et al.*, "Whole Genome Amplification from a Single Cell: Implications for Genetic Analysis", Proc. National Science USA, Vol. 89, pp. 5847-5851, July 1992
JS	David SIDRANSKY, *et al.*, "Clonal Expansion of p53 Mutant Cells is Associated with Brain Tumour Progression", Nature, February 27, 1992 Vol. 355, pp 846-847
JS	Alec J. Jeffreys, *et al.*, "Mutation Processes at Human Minisatellites", Electophoresis, pp. 1577-1585, 1995
JS	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, Vol. 66, pp. 129-141, 1994
JS	Paul M. LIZARDI, *et al.*, "Mutation Detection and Single-Molecule Counting Using Isothermal Rolling-Circle Amplification", Nature Genetics, Vol. 19, July 1998 pp 225-232
JS	~~W. NAVIDI, *et al.*, "Using PCR in Preimplantation Genetic Disease Diagnosis", Human Reproduction, Vol. 6, 1991~~
JS	~~Hongua LI, *et al.*, "Amplification and Analysis of DNA Sequences in Single Human Sperm and Diploid Cells" Nature, Vol. 335, September 29, 1988~~

EXAMINER Jeffrey Siei	DATE CONSIDERED 4/5/01

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	B	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

PTO-90C (Rev.11/00)

1- File Copy

Office Action Summary	Application No.	Applicant(s)	
	09/813,826	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 *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)☐ Acknowledgement 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

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 line19). 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 negatived 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 lines18-51 & 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 line30-35). 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 Tm of approximately 40C (Tm=[(A+T)x2C + (G+C) x4C](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 Tm 65C (see col. 28 line 54). 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.

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.

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

Notice of References Cited	Application/Control No. 09/613,826	Applicant(s)/Patent Under Reexamination VOGELSTEIN ET AL.	
	Examiner Jeffrey Siew	Art Unit ~~1636~~ 1637	Page 1 of 1

U.S. PATENT DOCUMENTS

*		Document Number Country Code-Number-Kind Code	Date MM-YYYY	Name	Classification	
	A	US-5,925,517 -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 Country Code-Number-Kind Code	Date MM-YYYY	Country	Name	Classification	
	N	- -					
	O	- -					
	P	- -					
	Q	- -					
	R	- -					
	S	- -					
	T	- -					

NON-PATENT DOCUMENTS

*		Include as applicable: Author, Title Date, Publisher, Edition or Volume, Pertinent Pages)
	U	Newton Esssential 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
D.Q.
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
Registration No. 32,141

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

#7

PTO-1449 (Modified) U.S. DEPARTMENT OF COMMERCE PATENT AND TRADEMARK OFFICE INFORMATION DISCLOSURE STATEMENT BY APPLICANT	ATTY. DOCKET NO. 01107.0003	SERIAL NUMBER ~~09/615,826~~ 61382
	APPLICANT Bert Vogelstein et al.	
	FILING DATE July 11, 2000	GROUP ART UNIT 1637

OIPE JUL 12 2001 PATENT & TRADEMARK OFFICE TC 1600

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.~~			
	~~5,670,325~~	~~9/23/1997~~	~~Lapidus et al.~~			
JS	~~6,030,137~~	~~2/1/2000~~	~~Lapidus et al.~~			

FOREIGN PATENT DOCUMENTS

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

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
	Monckton et al. "Minisatellite "Isoallele" Discrimination in Pseudohomozygotes 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 Fredman	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)	ATTY. DOCKET NO. 01107.00031	SERIAL NUMBER 09/613,826
U.S. DEPARTMENT OF COMMERCE PATENT AND TRADEMARK OFFICE	APPLICANT Bert Vogelstein et al.	
INFORMATION DISCLOSURE STATEMENT BY APPLICANT	FILING DATE July 11, 2000	GROUP ART UNIT 1637

U.S. PATENT DOCUMENTS

EXAMINER INITIAL	DOCUMENT NUMBER	DATE	NAME	CLASS	SUB CLASS	FILING DATE

FOREIGN PATENT DOCUMENTS

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

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

JS	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
JS	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	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.

PATENT

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

8/a
D.G.J
7/18/1

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

For: **DIGITAL AMPLIFICATION**

AMENDMENT

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.

a'

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 Tm 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 Tm of 54-56°C, and wherein the stem comprises 4 base pairs having a sequence 5'-CACG-3'.

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 Tm 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

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 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.

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.

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 umber 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 (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, annd 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 umber 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.

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.

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

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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:

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.

Application No.: 09/613,826

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):

- [X] 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).
- [X] 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).
- [X] 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 from of the "Sequence Listing" as required by 37 C.F.R. 1.821(e).
- [ ] 7. Other: _____

Applicant Must Provide:

- [X] An initial or substitute computer readable form (CRF) copy of the "Sequence Listing".
- [X] An initial or substitute paper copy of the "Sequence Listing", as well as an amendment directing its entry into the specification.
- [X] 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:

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☒ PATENT ☐ DESIGN B&W Ref. 1107.00031 Date 5/7/01
☐ HAND CARRY Group/Section _____ Bldg _____ Rm _____
Serial/Patent No. 09/813,926 Atty/Sec SAK/_____
Inventor VRAELSTEIN, et al. Client Jones Hannas
Title DIGITAL AMPLIFICATIONS

The following has been received in the U.S. Patent and Trademark Office on the date stamped hereon.

☐ ___ total pp Spec., including : # of Claims ____ (# of independent claims ____); ☐ Abstract
☐ Drawings : ☐ Formal ☐ Informal # of distinct sheets ____ : Figs ____
☐ Declaration/PoA : ☐ Executed ☐ Unexecuted
☐ Assignment w/PTO Cover Sheet
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☒ IDS w/PTO 1449 ☒ References ☐ w/Fee
☐ Preliminary Amendment
☐ Priority Claim (Foreign or U.S.Prov B&W# _____)
Country Appl # Date
☐ w/Foreign Priority Document(s)
☐ Application : ☐ CIP ☐ Continuation ☐ Divisional
Parent Ser No _____ B&W# _____
☐ U.S. Provisional _____ pp Spec/Clms, Cover Sht
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PATENT APPLICATION

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re application of:	)	
	)	
Bert Vogelstein, *et al.*	)	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

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

U.S. PATENT DOCUMENTS

EXAMINER INITIAL	DOCUMENT NUMBER	DATE	NAME	CLASS	SUB CLASS	FILING DATE
JS	5,670,325	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	Darren G. MONCKTON, *et al.*, "Minisatellite "Isoallele" Discrimination in Pseudohomozygotes by Single Molecule PCR and Variant Repeat Mapping", Genomics 11, pp. 465-467, 1991
	Gualberto RUANO, *et al.*, "Haplotype of Multiple Polymorphisms Resolved by Enzymatic Amplification of Single DNA Molecules", Proc. National Science USA, 1990
	W. NAVIDI, *et al.*, "Using PCR in Preimplantation Genetic Disease Diagnosis", Human Reproduction, Vol. 6, No. 6, pp. 836-849, 1991 Duplicate
	Honghua LI, *et al.*, "Amplification and Analysis of DNA Sequences in Single Human Sperm and Diploid Cells", Nature, Vol. 335, September 29, 1988
	Ramon PARSONS, *et al.*, "Mismatch Repair Deficiency in Phenotypically Normal Human Cells", Science, Vol. 268, May 5, 1995
	Lin ZHANG, *et al.*, "Whole Genome Amplification from a Single Cell: Implications for Genetic Analysis", Proc. National Science USA, Vol. 89, pp. 5847-5851, July 1992
	David SIDRANSKY, *et al.*, "Clonal Expansion of p53 Mutant Cells is Associated with Brain Tumour Progression", Nature, February 27, 1992
	Alec J. Jeffreys, *et al.*, "Mutation Processes at Human Minisatellites", Electophoresis, 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 Short Tandem Repeats (STR) Polymorphism", Forensic Science International, Vol. 66, pp. 129-141, 1994
	Paul M. LIZARDI, *et al.*, "Mutation Detection and Single-Molecule Counting Using Isothermal Rolling-Circle Amplification", Nature Genetics, Vol. 19, July 1998
	W. NAVIDI, *et al.*, "Using PCR in Preimplantation Genetic Disease Diagnosis", Human Reproduction, Vol. 6, 1991
JS	Honghua LI, *et al.*, "Amplification and Analysis of DNA Sequences in Single Human Sperm and Diploid Cells" Nature, Vol. 335, September 29, 1988

EXAMINER	DATE CONSIDERED
Jeffrey Siew	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.

B

Patent/Design ☐ PATENT ☐ DESIGN

☐ HAND CARRY Group/Section _____ Bldg _____ Rm ____

Serial No. 09/613,526 B&W # 010725031 Atty/Sec SAK/amc Date 12/15/00

Inventor [illegible] Client JHU

Title Digital Miniaturization

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☐ ___ total pp Spec., including : # of Claims ____ (# of independent claims _____); ☐ Abstract

☐ Foreign Priority on _____ Country, Appl # and Date

☐ Priority on U.S. Prov. _____ B&W# _____

☐ Application : ☐ CIP ☐ Continuation ☐ Divisional Parent Ser. No. _____ B&W# _____

☐ Provisional App ___ pp Spec/Clms; Cover Sht.

☐ Claim for Priority w/Priority Doc _____ Country, Appl. # and Date

☐ Petition for Extension til _____

☐ Amendment ☐ Response : OA dtd _____

☐ Request for Approval of Drawing Changes

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☐ Brief : ☐ Appeal & Fee ☐ Reply

Patent/Design ☐ PATENT ☐ DESIGN

☐ HAND CARRY Group/Section _____ Bldg _____ Rm ____

Serial No. 09/613,526 B&W # 010725031 Atty/Sec SAK/amc Date 12/15/00

Inventor [illegible] Client JHU

Title Digital Miniaturization

The following has been received in the U.S. Patent and Trademark Office on the date stamped hereon:

☐ ___ total pp Spec., including : # of Claims ____ (# of independent claims _____); ☐ Abstract

☐ Foreign Priority on _____ Country, Appl # and Date

☐ Priority on U.S. Prov. _____ B&W# _____

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☐ Provisional App ___ pp Spec/Clms; Cover Sht.

☒ Declaration/PoA : ☒ Executed ☐ Unexecuted

☐ Drawings : ☐ Formal ☐ Informal # of distinct sheets _____ Figs. _____

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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

Total Number of Pages in This Submission: 27

ENCLOSURES *(check all that apply)*

- ☐ Fee Transmittal Form
- ☐ Fee Attached
- ☒ Amendment / Response
- ☐ After Final
- ☐ Affidavits/declaration(s)
- ☐ Extension of Time Request
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- ☐ Information Disclosure Statement
- ☐ Certified Copy of Priority Document(s)
- ☐ Response to Missing Parts/ Incomplete Application
- ☐ Response to Missing Parts under 37 CFR 1.52 or 1.53
- ☐ Assignment Papers *(for an Application)*
- ☐ Drawing(s)
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- ☐ Appeal Communication to Group *(Appeal Notice, Brief, Reply Brief)*
- ☐ Proprietary Information
- ☐ Status Letter
- ☒ Other Enclosure(s) *(please identify below)*:
  Sequence Listing; Diskette Containing Sequence Listing; Copy of Notice to Comply; copies of 2 previously submitted IDS's; copies of references cited on IDS

Remarks: pages of references are in addition to Total Number of pages listed

SIGNATURE OF APPLICANT, ATTORNEY, OR AGENT

Firm or Individual name	Sarah A. Kagan 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: ____

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406795-1

SEQUENCE LISTING

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Kinzler, Kenneth W.

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PATENT

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re Application of

Bert Vogelstein, et. al.

Serial No. 09/613,826

Filing Date: July 11, 2000

) Group Art Unit: 1656

) Examiner: I Siew

) Docket No. 01107.00031

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For: DIGITAL AMPLIFICATION

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
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Application Number	09/613,826
Filing Date	July 11, 2000
First Named Inventor	VOGELSTEIN et al.
Group Art Unit	1656
Examiner Name	J. SIEW
Total Number of Pages in This Submission: 6	Attorney Docket Number: 01107.00031

ENCLOSURES *(check all that apply)*

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

Supplemental Submission
Copy of Reference

Remarks

SIGNATURE OF APPLICANT, ATTORNEY, OR AGENT

Firm or Individual name	Michelle Holmes-Son Reg. No. 47,680
Signature	Michelle Holmes-Son
Date	July 17, 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	

407556-1

UNITED STATE EPARTMENT OF COMMERCE
Patent and Trademark Office
Address: COMMISSIONER OF PATENTS AND TRADEMARKS
Washington, D.C. 20231

APPLICATION NO.	FILING DATE	FIRST NAMED INVENTOR	ATTORNEY DOCKET NO.
09/613,026	07/11/00	VOGELSTEIN	B 0110/0003

EXAMINER	
STEW, T	
ART UNIT	PAPER NUMBER
1656	10

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

DATE MAILED: 09/20/01

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

Commissioner of Patents and Trademarks

PTO-90C (Rev. 2/95)
*U.S. GPO: 2000-473-000/44602

1- File Copy

Office Action Summary	Application No.	Applicant(s)	
	09/813,626	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) ☐ Notice of References Cited (PTO-892)
2) ☐ Notice of Draftsperson's Patent Drawing Review (PTO-948)
3) ☒ Information Disclosure Statement(s) (PTO-1449) Paper No(s). 5.
4) ☐ Interview Summary (PTO-413) Paper No(s). _____ .
5) ☐ Notice of Informal Patent Application (PTO-152)
6) ☐ 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

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 Tm of 50-51°C and a stem consisting of 4 base pairs or one with a loop consisting of 19-20 base pairs and Tm 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 Tm of 50-51°C and stem comprising 4 base pairs nor a molecular beacon that a loop comprising 19-20 base pairs and Tm 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)x2C + (G+C) x4C] (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.

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,

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

Interview Summary	Application No.	Applicant(s)	
	09/613,826	VOGELSTEIN ET AL.	
	Examiner	Art Unit	
	Jeffrey Siew	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

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

DATE: 07/24/2001
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Kinzler, Kenneth W.
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<141> CURRENT FILING DATE: 2000-07-11
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PATENT APPLICATION: US/09/613,826 TIME: 11:12:17

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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

#12 DNW 10-3-01

1656

OIPE

RAW SEQUENCE LISTING DATE: 09/20/2001
PATENT APPLICATION: US/09/613,826 TIME: 17:06:04

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4 Kinzler, Kenneth W.
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Alpha or Numeric: Numeric
Application Class:
Application File Date: 07-11-2000
Art Unit: OIPE
Software Application: PatentIn
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Total Nucleotides: 145
Total Amino Acids: 0
Number of Errors: 0
Number of Warnings: 0
Number of Corrections: 0

MESSAGE SUMMARY

#13B/JRK
12-10-01
N.E.

PATENT

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re Application of	)
	) Group Art Unit: 1656
Bert Vogelstein, et. al.	)
	) Examiner: J Siew
Serial No. 09/613,826	) Box AF
	)
Filing Date: July 11, 2000	) 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 12/4/02 JSS

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 Tm 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 Tm 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 Tm 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 Tm

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'-CACGGGCCTGCTGAAAATGACTGCGTG-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 baess 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 reasonable 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 Tm of 50-51°C and stem comprising 4 base pairs nor a molecular beacon that a loop comprising 19-20 base pairs and Tm 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
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 Tm 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 Tm 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 Tm 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 Tm 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

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	Bert Vogelstein, et. al.
Group Art Unit	1656
Examiner Name	J. Siew
Total Number of Pages in This Submission: 13	Attorney Docket Number: 01107.00031

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ENCLOSURES *(check all that apply)*

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

Remarks

SIGNATURE OF APPLICANT, ATTORNEY, OR AGENT

Firm or Individual name	Michelle Holmes-Son, Registration No. 47,660
Signature	Michelle Holmes-Son
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

21907 7590 12/12/2001

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WASHINGTON, DC 20001

EXAMINER
SIEW, JEFFREY

ART UNIT	PAPER NUMBER
1656	14

DATE MAILED: 12/12/2001

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

PTO-90C (Rev. 07-01)

copy

Advisory Action	Application No.	Applicant(s)	
	09/613,826	VOGELSTEIN ET AL.	
	Examiner	Art Unit	
	Jeffrey Siew	1656	

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

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 fo 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 of 3. Applicant's reply has overcome the following rejection(s): the proposed amendment would over ocmver 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 Siew
3/23/02
copy filed 12/13/01

#15
2/13
1/31/02

PATENT APPLICATION

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
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.

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
(202) 508-9100

#15

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INFORMATION DISCLOSURE STATEMENT BY APPLICANT

(use as many sheets as necessary)

Sheet 1 of

JAN 23 2002 PATENT & TRADEMARK OFFICE

Complete If Known	
Application Number	09/613,826
Filing Date	July 11, 2000
First Named Inventor	Bert Vogelstein et al.
Group Art Unit	1656 1637
Examiner Name	J. Slew
Attorney Docket Number	01107.00031

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U.S. PATENT DOCUMENTS

Examiner Initials*	Cite No.[1]	Document Number Number - Kind Code[2] (if known)	Publication Date MM-DD-YYYY	Name of Patentee or Applicant of Cited Document	Pages, Columns, Lines, Where Relevant Passages or Relevant Figures Appear
JS		US-5,804,383	09-08-1998	Gruenert et al.	
JS		US- 5,658,663	01-12-1999	Nisson et al.	
		US-			
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FOREIGN PATENT DOCUMENTS

Examiner Initials*	Cite No.[1]	Foreign Patent Document Country Code[3] - Number[4] - Kind Code[5] (if known)	Publication Date MM-DD-YYYY	Name of Patentee or Applicant of Cited Document	Pages, Columns, Lines, Where Relevant Passages or Relevant Figures Appear	T[6]
JS		WO 95/13399	05-18-1995			
		EP 0643140 A	03-15-1995			
JS		WO 99/13113	03-18-1999			

Examiner Signature	Jeffrey Siew	Date Considered	3/23/02

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

[1] Applicant's unique citation designation number (optional). [2] See Kinds Codes of USPTO Patent Documents at www.uspto.gov or MPEP 901.04. [3] Enter Office that issued the document, by the two-letter code (WIPO Standard ST.3). [4] For Japanese patent documents, the indication of the year of the reign of the Emperor must precede the serial number of the patent document. [5] Kind of document by the appropriate symbols as indicated on the document under WIPO Standard ST. 16 if possible. [6] Applicant is to place a check mark here if English language Translation is attached.

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INFORMATION DISCLOSURE STATEMENT BY APPLICANT

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Sheet | of | 2

Complete if Known	
Application Number	09/613,826
Filing Date	July 11, 2000
First Named Inventor	Bert Vogelstein et al.
Group Art Unit	~~1635~~ 1637
Examiner Name	J. Siew
Attorney Docket Number	01107.00031

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OTHER PRIOR ART -- NON PATENT LITERATURE DOCUMENTS

Examiner Initials *	Cite No.[1]	Include name of the author (in CAPITAL LETTERS), title of the article (when appropriate), title of the item (book, magazine, journal, serial, symposium, catalog, etc.), date, page(s), volume-issue number(s), publisher, city and/or country where published.	T[2]
		A. PIATEK et al., "Molecular Beacon Sequence Analysis for Detecting Drug Resistance in Mycobacterium Tuberculosis", Nature Biotechnology, April 1998, pp. 359-363, Vol. 16, 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., "Multiplex 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	
		B. VOGELSTEIN et al., "Digital PCR", Proceedings of the National Academy of Sciences of the United States, August 3, 1999, pp. 9236-9241, 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	Jeffrey Siew	Date Considered	3/18/02

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

[1] Unique citation designation number (optional). [2] 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.

OIPE JC18 JAN 23 2002 PATENT & TRADEMARK OFFICE

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Complete If Known	
Application Number	09/613,826
Filing Date	July 11, 2000
First Named Inventor	Bert Vogelstein et al.
Examiner Name	J. Siew
Group / Art Unit	1656
Attorney Docket No.	01107.00031

TOTAL AMOUNT OF PAYMENT ($) 180

METHOD OF PAYMENT (check all that apply)

☐ Check ☐ Credit card ☐ Money Order ☐ Other ☐ None

☒ Deposit Account:

Deposit Account Number: Banner & Witcoff, Ltd.

Deposit Account Name: 19-0733

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

1. BASIC FILING FEE

Large Entity Fee Code	Large Entity Fee ($)	Small Entity Fee Code	Small Entity Fee ($)	Fee Description	Fee Paid
101	740	201	370	Utility filing fee	
106	330	206	165	Design filing fee	
107	510	207	255	Plant filing fee	
108	740	208	370	Reissue filing fee	
114	160	214	80	Provisional filing fee	

SUBTOTAL (1) ($) 0

2. EXTRA CLAIM FEES

			Extra Claims		Fee from below	=	Fee Paid
Total Claims		-20 **	= 0	X		=	0
Independent Claims		-3 **	= 0	X		=	0
Multiple Dependent				X		=	0

Large Entity Fee Code	Large Entity Fee ($)	Small Entity Fee Code	Small Entity Fee ($)	Fee Description
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

SUBTOTAL (2) ($) 0

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

FEE CALCULATION (continued)

3. ADDITIONAL FEES

Large Entity Fee Code	Large Entity Fee ($)	Small Entity Fee Code	Small Entity Fee ($)	Fee Description	Fee Paid
105	130	205	65	Surcharge - late filing fee or oath	
127	60	227	25	Surcharge - late provisional filing fee or cover sheet.	
139	130	139	130	Non-English specification	
147	2,520	147	2,520	For filing a request for reexamination	
112	920*	112	920*	Requesting publication of SIR prior to Examiner action	
113	1,840*	113	1,840*	Requesting publication of SIR after Examiner action	
115	110	215	55	Extension for reply within first month	
116	400	216	200	Extension for reply within second month	
117	920	217	460	Extension for reply within third month	
118	1,440	218	720	Extension for reply within fourth month	
128	1,960	228	980	Extension for reply within fifth month	
119	320	219	160	Notice of Appeal	
120	320	220	160	Filing a brief in support of an appeal	
121	280	221	140	Request for oral hearing	
138	1,510	138	1,510	Petition to institute a public use proceeding	
140	110	240	55	Petition to revive – unavoidable	
141	1,280	241	640	Petition to revive – unintentional	
142	1,280	242	640	Utility issue fee (or reissue)	
143	460	243	230	Design issue fee	
144	620	244	310	Plant issue fee	
122	130	122	130	Petitions to the Commissioner	
123	50	123	50	Processing fee under 37 CFR 1.17 (q)	
126	180	126	180	Submission of Information Disclosure Stmt	180
581	40	581	40	Recording each patent assignment per property (times number of properties)	
146	740	246	370	Filing a submission after final rejection (37 CFR § 1.129(a))	
149	740	249	370	For each additional invention to be examined (37 CFR § 1.129(b))	
179	740	279	370	Request for Continued Examination (RCE)	
169	900	169	900	Request for expedited examination of a design application	
				Other fee (specify)	

*Reduced by Basic Filing Fee Paid SUBTOTAL (3) ($) 180

SUBMITTED BY				Complete (if applicable)	
Name (Print/Type)	Michelle L. Holmes-Son	Registration No. Attorney/Agent	47,660	Telephone	202-508-9100
Signature	Michelle L. Holmes-Son			Date	January 23, 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-2038.

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

PTO/SB/21 (08-00)

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

Approved for use through 10/31/2002. OMB 0651-0031
U.S. Patent and Trademark Office: U.S. DEPARTMENT OF COMMERCE
Under the Paperwork Reduction Act of 1995, no persons are required to respond to a collection of information unless it displays a valid OMB control number.

TRANSMITTAL FORM

(to be used for all correspondence after initial filing)

JAN 23 2002 PATENT & TRADEMARK OFFICE

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
Attorney Docket Number	01107.00031

Total Number of Pages in This Submission

RECEIVED JAN 28 2002 TECH CENTER 1600/2900

ENCLOSURES *(check all that apply)*

- [x] Fee Transmittal Form
  - [ ] Fee Attached
- [ ] Amendment / Response
  - [ ] After Final
  - [ ] Affidavits/declaration(s)
- [ ] Extension of Time Request
- [ ] Express Abandonment Request
- [x] Information Disclosure Statement
- [ ] Certified Copy of Priority Document(s)
- [ ] Response to Missing Parts/ Incomplete Application
  - [ ] Response to Missing Parts under 37 CFR 1.52 or 1.53
- [ ] Assignment Papers *(for an Application)*
- [ ] Drawing(s)
- [ ] Licensing-related Papers
- [ ] Petition
- [ ] Petition to Convert to a Provisional Application
- [ ] Power of Attorney, Revocation Change of Correspondence Address
- [ ] Terminal Disclaimer
- [ ] Request for Refund
- [ ] CD, Number of CD(s) ____
- [ ] After Allowance Communication to Group
- [ ] Appeal Communication to Board of Appeals and Interferences
- [ ] Appeal Communication to Group *(Appeal Notice, Brief, Reply Brief)*
- [ ] Proprietary Information
- [ ] Status Letter
- [x] Other Enclosure(s) *(please identify below)*:
  PTO-1449 (w/references)

Remarks

SIGNATURE OF APPLICANT, ATTORNEY, OR AGENT

Firm or Individual name	Michelle L. Holmes-Son, Registration No. 47,660
Signature	Michelle L Holmes-Son
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	

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.

PTO/SB/22 (10-00)
Approved for use through 10/31/2002. OMB 0651-0031
U.S. Patent and Trademark Office; U.S. DEPARTMENT OF COMMERCE
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#16
2/26/02

PETITION FOR EXTENSION OF TIME UNDER 37 CFR 1.136(a)

Docket Number (Optional)
001107.00031

O I P E
FEB 20 2002
PATENT & TRADEMARK OFFICE

RECEIVED
FEB 25 2002
TECH CENTER 1600/2900

In re Application of Bert VOGELSTEIN, et a.	
Application Number 09/613,826	Filed July 11, 2000
For Digital Amplification	
Group Art Unit 1656	Examiner 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):

- ☐ One month (37 CFR 1.17(a)(1)) $____
- ☒ Two months (37 CFR 1.17(a)(2)) $400.00
- ☐ Three months (37 CFR 1.17(a)(3)) $____
- ☐ Four months (37 CFR 1.17(a)(4)) $____
- ☐ 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). ______ .

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.

February 20, 2002
Date

Michelle L. Holmes-Son
Signature

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

02/27/2002 ... 00000054 190733 09613826
... 200.00 CH

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.

Burden Hour Statement: This form is estimated to take 0.1 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.

Approved for use through 10/31/2002. OMB 0651-0032
U.S. Patent and Trademark Office: U.S. DEPARTMENT OF COMMERCE
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FEE TRANSMITTAL for FY 2002

OIPE FEB 20 2002 PATENT & TRADEMARK OFFICE

Patent fees are subject to annual revision.

RECEIVED FEB 25 2002 TECH CENTER 1600/2900

TOTAL AMOUNT OF PAYMENT ($) 200

Complete If Known	
Application Number	09/813,926
Filing Date	July 11, 2000
First Named Inventor	Bert VOGELSTEIN, et al.
Examiner Name	Jeffrey Siew
Group / Art Unit	1656
Attorney Docket No.	001107 00031

METHOD OF PAYMENT (check all that apply)

☐ Check ☐ Credit card ☐ Money Order ☐ Other ☐ None

☐ Deposit Account:

Deposit Account Number: 19-0733

Deposit Account Name: Banner & Witcoff, 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

1. BASIC FILING FEE

Large Entity Fee Code	Large Entity Fee ($)	Small Entity Fee Code	Small Entity Fee ($)	Fee Description	Fee Paid
101	740	201	370	Utility filing fee	
106	330	206	165	Design filing fee	
107	510	207	255	Plant filing fee	
108	740	208	370	Reissue filing fee	
114	160	214	80	Provisional filing fee	

SUBTOTAL (1) ($) 0

2. EXTRA CLAIM FEES

			Extra Claims		Fee from below	=	Fee Paid
Total Claims		-20 **	0	X		=	0
Independent Claims		-3 **	0	X		=	0
Multiple Dependent				X		=	0

Large Entity Fee Code	Large Entity Fee ($)	Small Entity Fee Code	Small Entity Fee ($)	Fee Description
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

SUBTOTAL (2) ($) 0

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

FEE CALCULATION (continued)

3. ADDITIONAL FEES

Large Entity Fee Code	Large Entity Fee ($)	Small Entity Fee Code	Small Entity Fee ($)	Fee Description	Fee Paid
105	130	205	65	Surcharge - late filing fee or oath	
127	50	227	25	Surcharge - late provisional filing fee or cover sheet.	
139	130	139	130	Non-English specification	
147	2,520	147	2,520	For filing a request for reexamination	
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117	920	217	460	Extension for reply within third month	
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138	1,510	138	1,510	Petition to institute a public use proceeding	
140	110	240	55	Petition to revive – unavoidable	
141	1,280	241	640	Petition to revive – unintentional	
142	1,280	242	640	Utility issue fee (or reissue)	
143	460	243	230	Design issue fee	
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126	180	126	180	Submission of Information Disclosure Stmt	
581	40	581	40	Recording each patent assignment per property (times number of properties)	
146	740	246	370	Filing a submission after final rejection (37 CFR § 1.129(a))	
149	740	249	370	For each additional invention to be examined (37 CFR § 1.129(b))	
179	740	279	370	Request for Continued Examination (RCE)	
169	900	169	900	Request for expedited examination of a design application	
Other fee (specify)					

*Reduced by Basic Filing Fee Paid SUBTOTAL (3) ($) 200

SUBMITTED BY				Complete (if applicable)	
Name (Print/Type)	Michelle L. Holmes-Son	Registration No. Attorney/Agent	47,860	Telephone	(202) 508-9100
Signature	Michelle L. Holmes-Son			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-2038.

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

AF/1656 $

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

PTO/SB/21 (08-00)
Approved for use through 10/31/2002. OMB 0651-0031
U.S. Patent and Trademark Office: U.S. DEPARTMENT OF COMMERCE
Under the Paperwork Reduction Act of 1995, no persons are required to respond to a collection of information unless it displays a valid OMB control number.

TRANSMITTAL FORM

(to be used for all correspondence after initial filing)

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
Attorney Docket Number	001107.00031

Total Number of Pages in This Submission

ENCLOSURES *(check all that apply)*

- ☒ Fee Transmittal Form
- ☐ Fee Attached
- ☒ Amendment / Response
- ☒ After Final
- ☐ Affidavits/declaration(s)
- ☒ Extension of Time Request
- ☐ Express Abandonment Request
- ☐ Information Disclosure Statement
- ☐ Certified Copy of Priority Document(s)
- ☐ Response to Missing Parts/ Incomplete Application
- ☐ Response to Missing Parts under 37 CFR 1.52 or 1.53
- ☐ Assignment Papers *(for an Application)*
- ☐ Drawing(s)
- ☐ Licensing-related Papers
- ☐ Petition
- ☐ Petition to Convert to a Provisional Application
- ☐ Power of Attorney, Revocation Change of Correspondence Address
- ☐ Terminal Disclaimer
- ☐ Request for Refund
- ☐ CD, Number of CD(s) ____
- ☐ After Allowance Communication to Group
- ☐ Appeal Communication to Board of Appeals and Interferences
- ☐ Appeal Communication to Group *(Appeal Notice, Brief, Reply Brief)*
- ☐ Proprietary Information
- ☐ Status Letter
- ☒ Other Enclosure(s) *(please identify below)*:
  - Sequence Listing
  - Computer Readable Disk

Remarks

SIGNATURE OF APPLICANT, ATTORNEY, OR AGENT

Firm or Individual name	Michelle L. Holmes-Son, Reg. No. 47,860
Signature	Michelle L. Holmes-Son
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 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.

PATENT

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

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

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

1

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

1/10

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).

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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 +/- 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.

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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Interview Summary	Application No.	Applicant(s)	
	09/613,626	VOGELSTEIN ET AL.	
	Examiner	Art Unit	
	Jeffrey Slow	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.

Jeffrey Slew
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

1637

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

Input Set : A:\107.31.ST25.txt
Output Set: N:\CRF3\03012002\I613826A.raw

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: 09/613,826A
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
16 <160> NUMBER OF SEQ ID NOS: 15
18 <170> SOFTWARE: PatentIn version 3.1
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RAW SEQUENCE LISTING DATE: 03/01/2002
PATENT APPLICATION: US/09/613,826A TIME: 15:29:37

Input Set : A:\107.31.ST25.txt
Output Set: N:\CRF3\03012002\I613826A.raw

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

Input Set : A:\107.31.ST25.txt
Output Set: N:\CRF3\03012002\I613826A.raw

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

Input Set : A:\107.31.ST25.txt
Output Set: N:\CRF3\03012002\I613826A.raw

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:_____

Interview Summary	Application No.	Applicant(s)	
	09/613,829	TAKESHITA ET AL.	
	Examiner	Art Unit	
	Jeffrey Siew	1656	

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

(1) *Jeffrey Siew*. (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 aa 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

Notice of Allowability	Application No. 09/813,829	Applicant(s) TAKESHITA ET AL.
	Examiner Jeffrey Siew	Art Unit 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 Draftsperson'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 Draftsperson.

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)

1☐ Notice of References Cited (PTO-892)
3☒ Notice of Draftperson's Patent Drawing Review (PTO-948)
5☐ Information Disclosure Statements (PTO-1449), Paper No. _____.
7☐ Examiner's Comment Regarding Requirement for Deposit of Biological Material
2☐ Notice of Informal Patent Application (PTO-152)
4☒ Interview Summary (PTO-413), Paper No. 18+20
6☐ Examiner's Amendment/Comment
8☒ Examiner's Statement of Reasons for Allowance
9☐ Other

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 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.

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.

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

Mail 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. 20231
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	435-006000

DATE MAILED: 03/24/2002

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	06/24/2002

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.

PTOL-85 (REV. 07-01) Approved for use through 01/31/2004.

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 complet 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" f maintenance fee notifications.

CURRENT CORRESPONDENCE ADDRESS (Note: Legibly mark-up with any corrections or use Block 1)

22907 7590 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 assignmen 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.

(Depositor's nam
(Signatur
(Dat

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

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.

☐ "Fee Address" indication (or "Fee Address" Indication form PTO/SB/47) attached

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

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

☐ Issue Fee
☐ Publication Fee
☐ Advance Order - # of Copies _____

4b. Payment of Fee(s):

☐ A check in the amount of the fee(s) is enclosed.
☐ Payment by credit card. Form PTO-2038 is attached.
☐ 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).

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 to 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 COMPLETED FORMS TO THIS ADDRESS. SEND FEES AND THIS FORM TO: Box Issue Fee, Assistant Commissioner for Patents, Washington, D.C. 20231

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

TRANSMIT THIS FORM WITH FEE(S)

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. 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 7590 03/24/2002

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

EXAMINER	
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. 09/613,829	Applicant(s) TAKESHITA ET AL.	
	Examiner Jeffrey Siew	Art Unit 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.
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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 Draftsperson's Patent Drawing Review (PTO-948) attached
      1) ☐ hereto or 2) ☒ to Paper No. 21.
   (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. _____.

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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)

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3☒ Notice of Draftperson's Patent Drawing Review (PTO-948)
5☐ Information Disclosure Statements (PTO-1449), Paper No. _____.
7☐ Examiner's Comment Regarding Requirement for Deposit of Biological Material
2☐ Notice of Informal Patent Application (PTO-152)
4☒ Interview Summary (PTO-413), Paper No. 18 & 20
6☐ Examiner's Amendment/Comment
8☒ Examiner's Statement of Reasons for Allowance
9☐ Other

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 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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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

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

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

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.

PTOL-85 (REV. 07-01) Approved for use through 01/31/2004.

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 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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22907 7590 03/26/2002

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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.

(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
SIBW, 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.
- ☐ "Fee Address" indication (or "Fee Address" Indication form PTO/SB/47) attached.

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

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

- ☐ Issue Fee
- ☐ Publication Fee
- ☐ Advance Order - # of Copies _____

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

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 to 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 COMPLETED 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 DEPARTMENT OF COMMERCE
United States Patent and Trademark Office
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Washington, D.C. 20231
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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

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

EXAMINER	
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)

PTOL-85 (REV. 07-01) Approved for use through 01/31/2004.

PART B - FEE(S) TRANSMITTAL

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22907 7590 05/26/2002

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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	01107.00051	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	08/26/2002

EXAMINER	ART UNIT	CLASS-SUBCLASS
SHEW, 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.

☐ "Fee Address" indication (or "Fee Address" Indication form PTO/SB/47) attached.

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 Banner & Witcoff, Ltd.
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: 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 ☒ corporation or other private group entity ☐ government

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

☒ Issue Fee
☐ Publication Fee
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☐ A check in the amount of the fee(s) is enclosed.
☐ Payment by credit card. Form PTO-2038 is attached.
☒ The Commissioner is hereby authorized by charge the required fee(s), or credit any overpayment, to Deposit Account Number 19-0733 (enclose an extra copy of this form).

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 to the application identified above.

(Authorized Signature) _____ (Date)
Michelle Holmes-Son, Reg. No. 47,660 May 21, 2002

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.

[redacted]

05/22/2002 TDEMMIE 00000060 190733 09613826
01 FC:242 640.00 CH
02 FC:1561 30.00 CH

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PATENT APPLICATION

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
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.

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
(202) 508-9100

PTO/SB/08A (10-01)
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U.S. Patent and Trademark Office: U.S. DEPARTMENT OF COMMERCE
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Substitute for form 1449A/PTO

INFORMATION DISCLOSURE STATEMENT BY APPLICANT

(use as many sheets as necessary)

Sheet 1 of 2

Complete if Known	
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
Attorney Docket Number	01107.00031

U.S. PATENT DOCUMENTS

Examiner Initials*	Cite No.[1]	Document Number: Number - Kind Code[2] (if known)	Publication Date MM-DD-YYYY	Name of Patentee or Applicant of Cited Document	Pages, Columns, Lines, Where Relevant Passages or Relevant Figures Appear
		US-5,804,383	09-08-1998	Gruenert et al.	
		US-5,858,653	01-12-1999	Nilsson et al.	
		US-			
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		US-			
		US-			

FOREIGN PATENT DOCUMENTS

Examiner Initials*	Cite No.[1]	Foreign Patent Document: Country Code[3] - Number[4] - Kind Code[5] (if known)	Publication Date MM-DD-YYYY	Name of Patentee or Applicant of Cited Document	Pages, Columns, Lines, Where Relevant Passages or Relevant Figures Appear	T[6]
		WO 95/13399	05-18-1995			
		EP 0643140 A	03-15-1995			
		WO 99/13113	03-18-1999			

Examiner Signature		Date Considered	4/15/02

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

[1] Applicant's unique citation designation number (optional). [2] See Kinds Codes of USPTO Patent Documents at www.uspto.gov or MPEP 901.04. [3] Enter Office that issued the document, by the two-letter code (WIPO Standard ST.3). [4] For Japanese patent documents, the indication of the year of the reign of the Emperor must precede the serial number of the patent document. [5] Kind of document by the appropriate symbols as indicated on the document under WIPO Standard ST. 16 if possible. [6] 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.

PTO/SB/08B(10-01)
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U.S. Patent and Trademark Office: U.S. DEPARTMENT OF COMMERCE
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Substitute for form 1449A/PTO

INFORMATION DISCLOSURE STATEMENT BY APPLICANT

(use as many sheets as necessary)

Sheet 2 of 2

Complete If Known	
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
Attorney Docket Number	01107.00031

OTHER PRIOR ART – NON PATENT LITERATURE DOCUMENTS

Examiner Initials *	Cite No.[1]	Include name of the author (in CAPITAL LETTERS), title of the article (when appropriate), title of the item (book, magazine, journal, serial, symposium, catalog, etc.), date, page(s), volume-issue number(s), publisher, city and/or country where published.	T[2]
JS		A. PIATEK et al., "Molecular Beacon Sequence Analysis for Detecting Drug Resistance in Mycobacterium Tuberculosis", Nature Biotechnology, April 1998, pp. 359-363, Vol. 16, 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., "Multiplex 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. 101-191, Vol. 268, No. 2	
		B. VOGELSTEIN et al., "Digital PCR", Proceedings of the National Academy of Sciences of the United States, August 3, 1999, pp. 9236-9241, Vol. 96, No. 16	
JS		K. D.E. EVERETT et al, "Identification of nine species of the Chlamydiaceae Using PCR-RFLP", April 1999, pp. 903-913, Vol. 49, No. 2	

Examiner Signature	J Siew	Date Considered	4/15/02

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

[1] Unique citation designation number (optional). [2] 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.

PTO/SB/17 (10-01)
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FEE TRANSMITTAL for FY 2002

Patent fees are subject to annual revision.

TOTAL AMOUNT OF PAYMENT ($) 160

Complete if Known	
Application Number	09/613,626
Filing Date	July 11, 2000
First Named Inventor	Bert Vogelstein et al.
Examiner Name	J. Siew
Group / Art Unit	1656
Attorney Docket No.	01107.00031

METHOD OF PAYMENT (check all that apply)

☐ Check ☐ Credit card ☐ Money Order ☐ Other ☐ None

☒ Deposit Account:

Deposit Account Number: Banner & Witcoff, Ltd.

Deposit Account Name: 19-0733

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

1. BASIC FILING FEE

Large Entity Fee Code	Large Entity Fee ($)	Small Entity Fee Code	Small Entity Fee ($)	Fee Description	Fee Paid
101	740	201	370	Utility filing fee	
106	330	206	165	Design filing fee	
107	510	207	255	Plant filing fee	
108	740	208	370	Reissue filing fee	
114	150	214	80	Provisional filing fee	
SUBTOTAL (1)					($) 0

2. EXTRA CLAIM FEES

			Extra Claims		Fee from below		Fee Paid
Total Claims		-20** =	0	X		=	0
Independent Claims		-3** =	0	X		=	0
Multiple Dependents				X		=	0

Large Entity Fee Code	Large Entity Fee ($)	Small Entity Fee Code	Small Entity Fee ($)	Fee Description
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
SUBTOTAL (2)				($) 0

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

FEE CALCULATION (continued)

3. ADDITIONAL FEES

Large Entity Fee Code	Large Entity Fee ($)	Small Entity Fee Code	Small Entity Fee ($)	Fee Description	Fee Paid
105	130	205	65	Surcharge - late filing fee or oath	
127	50	227	25	Surcharge - late provisional filing fee or cover sheet.	
139	130	139	130	Non-English specification	
147	2,520	147	2,520	For filing a request for reexamination	
112	920*	112	920*	Requesting publication of SIR prior to Examiner action	
113	1,840*	113	1,840*	Requesting publication of SIR after Examiner action	
115	110	215	55	Extension for reply within first month	
116	400	216	200	Extension for reply within second month	
117	920	217	460	Extension for reply within third month	
118	1,440	218	720	Extension for reply within fourth month	
128	1,960	228	980	Extension for reply within fifth month	
119	330	219	165	Notice of Appeal	
120	330	220	165	Filing a brief in support of an appeal	
121	280	221	140	Request for oral hearing	
138	1,510	138	1,510	Petition to institute a public use proceeding	
140	110	240	55	Petition to revive – unavoidable	
141	1,280	241	640	Petition to revive – unintentional	
142	1,280	242	640	Utility issue fee (or reissue)	
143	480	243	240	Design issue fee	
144	620	244	310	Plant issue fee	
122	130	122	130	Petitions to the Commissioner	
123	50	123	50	Processing fee under 37 CFR 1.17 (q)	
126	180	126	180	Submission of Information Disclosure Stmt	160
581	40	581	40	Recording each patent assignment per property (times number of properties)	
146	740	246	370	Filing a submission after final rejection (37 CFR § 1.129(a))	
149	740	249	370	For each additional invention to be examined (37 CFR § 1.129(b))	
179	740	279	370	Request for Continued Examination (RCE)	
169	900	169	900	Request for expedited examination of a design application	
Other fee (specify)					
*Reduced by Basic Filing Fee Paid				SUBTOTAL (3)	($) 160

SUBMITTED BY				Complete (if applicable)	
Name (Print/Type)	Michelle L. Holmes-Sloan	Registration No. Attorney/Agent	47,630	Telephone	202-508-9100
Signature				Date	January 23, 2002

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PTO/SB/21 (09-00)

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U.S. Patent and Trademark Office: U.S. DEPARTMENT OF COMMERCE

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TRANSMITTAL FORM

(to be used for all correspondence after initial filing)

Total Number of Pages in This Submission

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
Attorney Docket Number	01107.00031

ENCLOSURES *(check all that apply)*

- [X] Fee Transmittal Form
  - [ ] Fee Attached
- [ ] Amendment / Response
  - [ ] After Final
  - [ ] Affidavits/declaration(s)
- [ ] Extension of Time Request
- [ ] Express Abandonment Request
- [X] Information Disclosure Statement
- [ ] Certified Copy of Priority Document(s)
- [ ] Response to Missing Parts/ Incomplete Application
  - [ ] Response to Missing Parts under 37 CFR 1.52 or 1.53
- [ ] Assignment Papers *(for an Application)*
- [ ] Drawing(s)
- [ ] Licensing-related Papers
- [ ] Petition
- [ ] Petition to Convert to a Provisional Application
- [ ] Power of Attorney, Revocation Change of Correspondence Address
- [ ] Terminal Disclaimer
- [ ] Request for Refund
- [ ] CD, Number of CD(s) ____
- [ ] After Allowance Communication to Group
- [ ] Appeal Communication to Board of Appeals and Interferences
- [ ] Appeal Communication to Group *(Appeal Notice, Brief, Reply Brief)*
- [ ] Proprietary Information
- [ ] Status Letter
- [X] Other Enclosure(s) *(please identify below)*: PTO-1449 (w/references)

Remarks

SIGNATURE OF APPLICANT, ATTORNEY, OR AGENT

Firm or Individual name	Michelle L. Holmes-Sen, Registration No. 47,660
Signature	
Date	January 23, 2002

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INTELLECTUAL PROPERTY LAW

1001 G STREET, N.W.
WASHINGTON, D.C. 20001-4597

TEL: 202.508.9100
FAX: 202.508.9299
www.bannerwitcoff.com

FACSIMILE TRANSMITTAL SHEET

TO:	FROM:
Jeffrey Slew	Michelle L. Holmes-Son
COMPANY:	DATE:
United States Patent and Trademark Office	April 15, 2002
FAX NUMBER:	TOTAL NO. OF PAGES:
(703)-746-5178	7, including this cover sheet
RE:	OUR REFERENCE NO.:
U.S. Serial Number: 09/813,826	01107.00031

If you do not receive all page(s) or have any problems receiving this transmission, please call:

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 29, 2002. Please return by fax after you are satisfied with your review of the document.

Yours truly,

Michelle L. Holmes-Son (47,660)

#23 Formal Drwgs

PATENT

6/17/02

#25 - Drwgs

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re Application of	)
	) Group Art Unit: 1637
Bert Vogelstein, et. al.	)
	) Examiner: Jeffrey Siew
Serial No. 09/613,826	)
	)
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:

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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
Registration No. 47,660

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

03/02

FIG. 1A

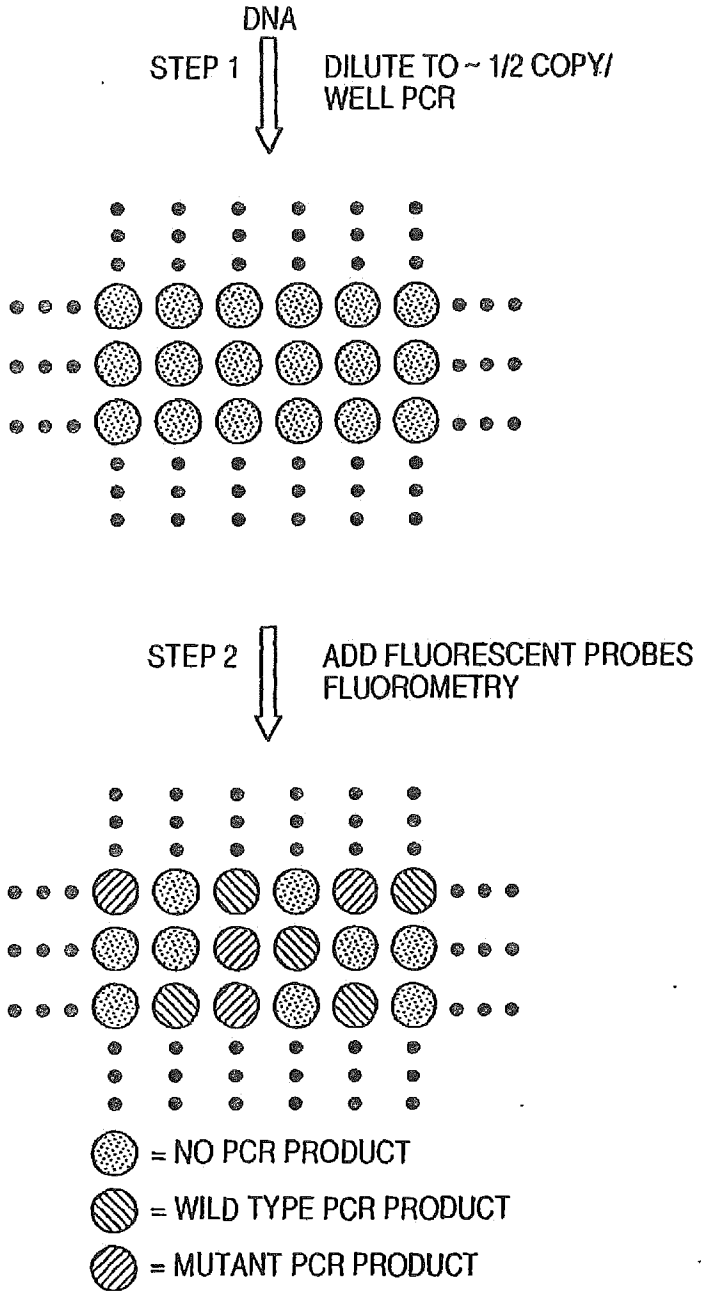
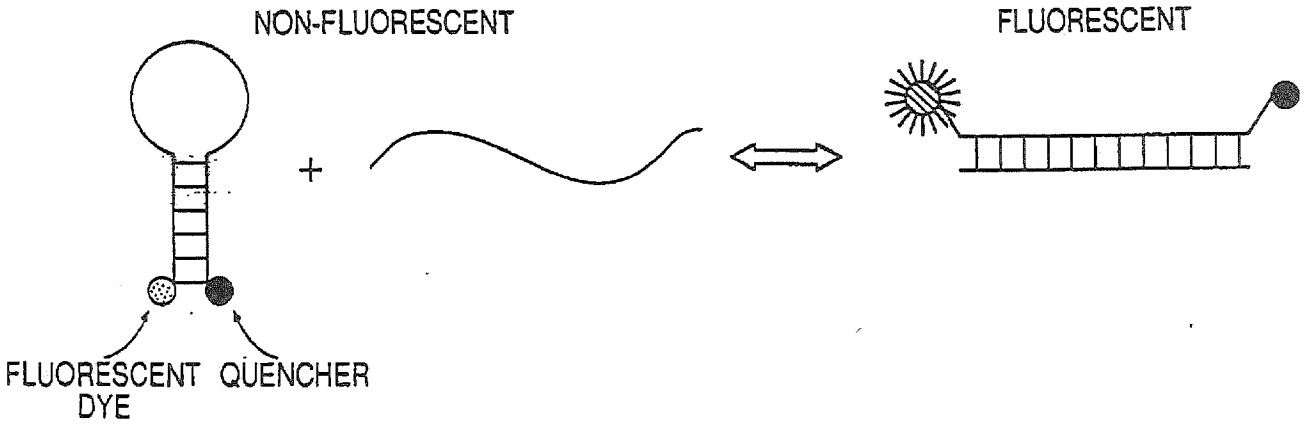

DNA
STEP 1
DILUTE TO ~ 1/2 COPY/ WELL PCR
STEP 2
ADD FLUORESCENT PROBES FLUOROMETRY
= NO PCR PRODUCT
= WILD TYPE PCR PRODUCT
= MUTANT PCR PRODUCT

FIG. 1B



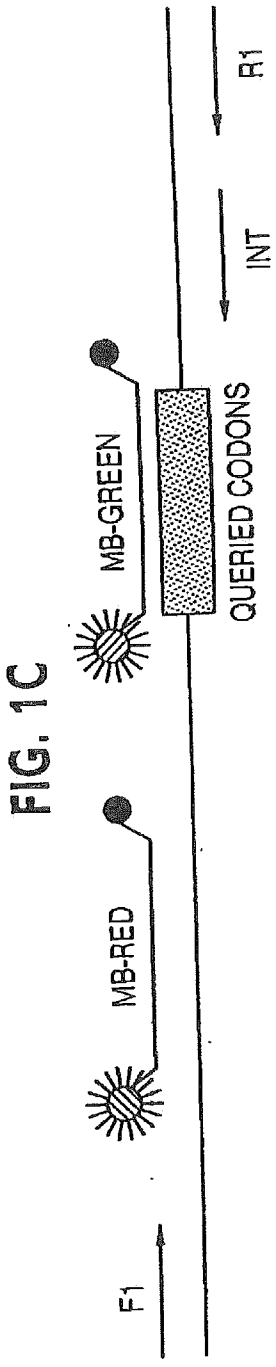
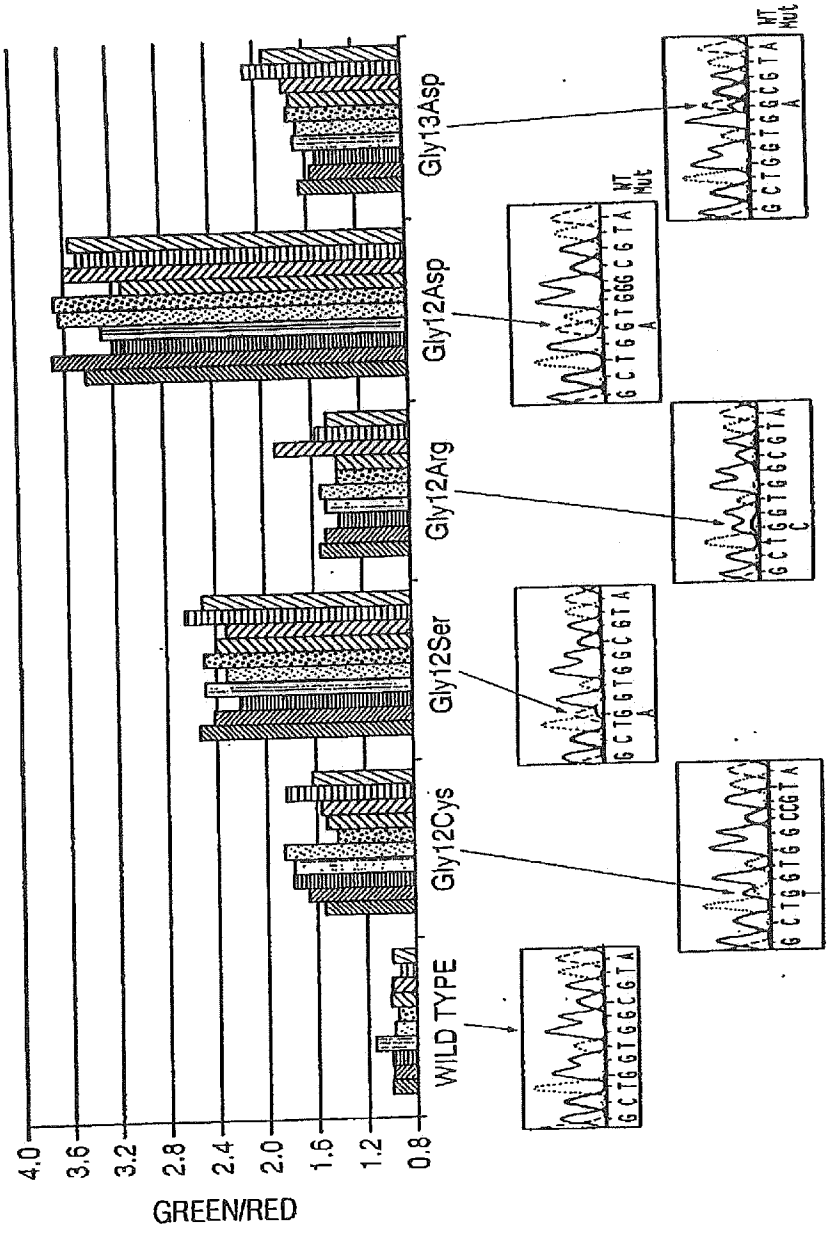


FIG. 1C

FIG. 2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3.6
3.2
2.8
2.4
2.0
1.6
1.2
0.8
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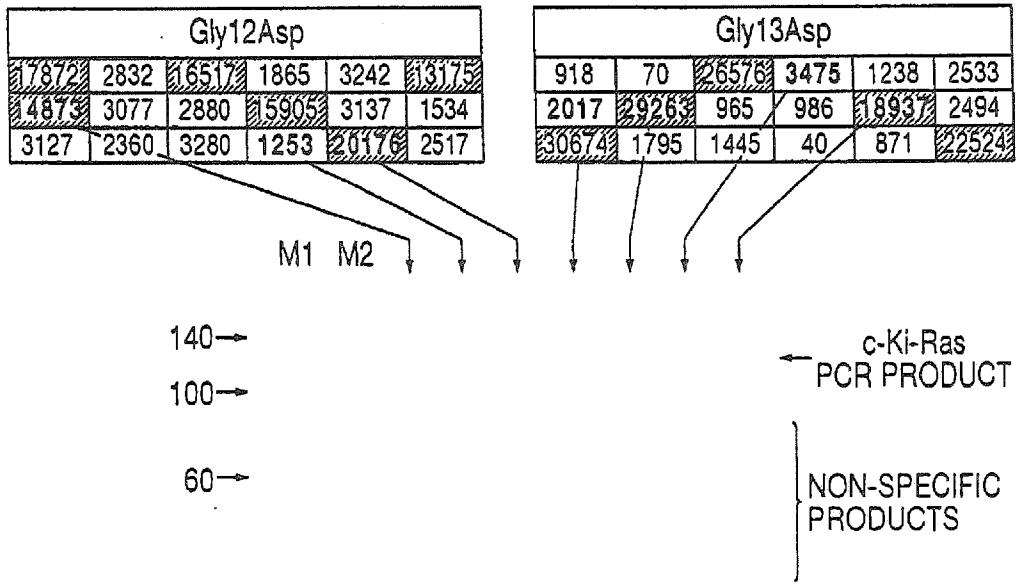
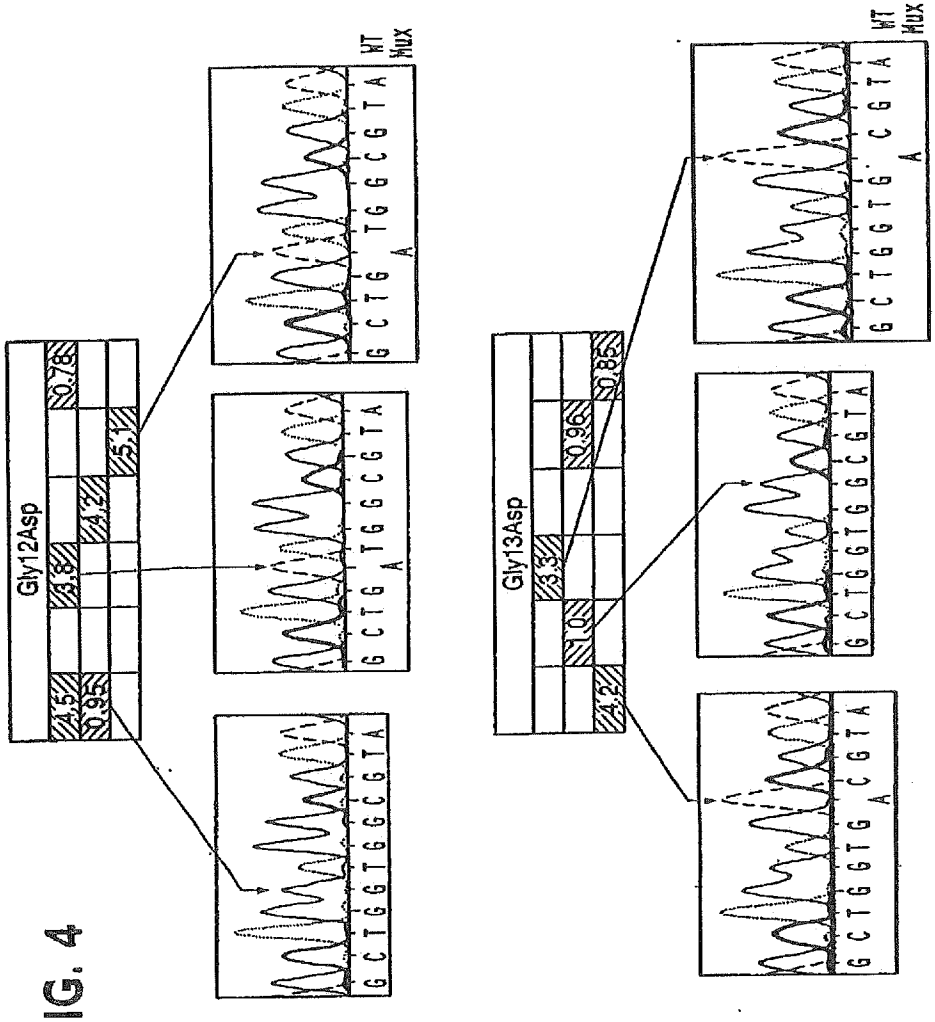
Gly12Ser
Gly12Arg
Gly12Asp
Gly13Asp
WT
Mut

FIG. 3

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0.95
3.8
4.2
5.1
0.78
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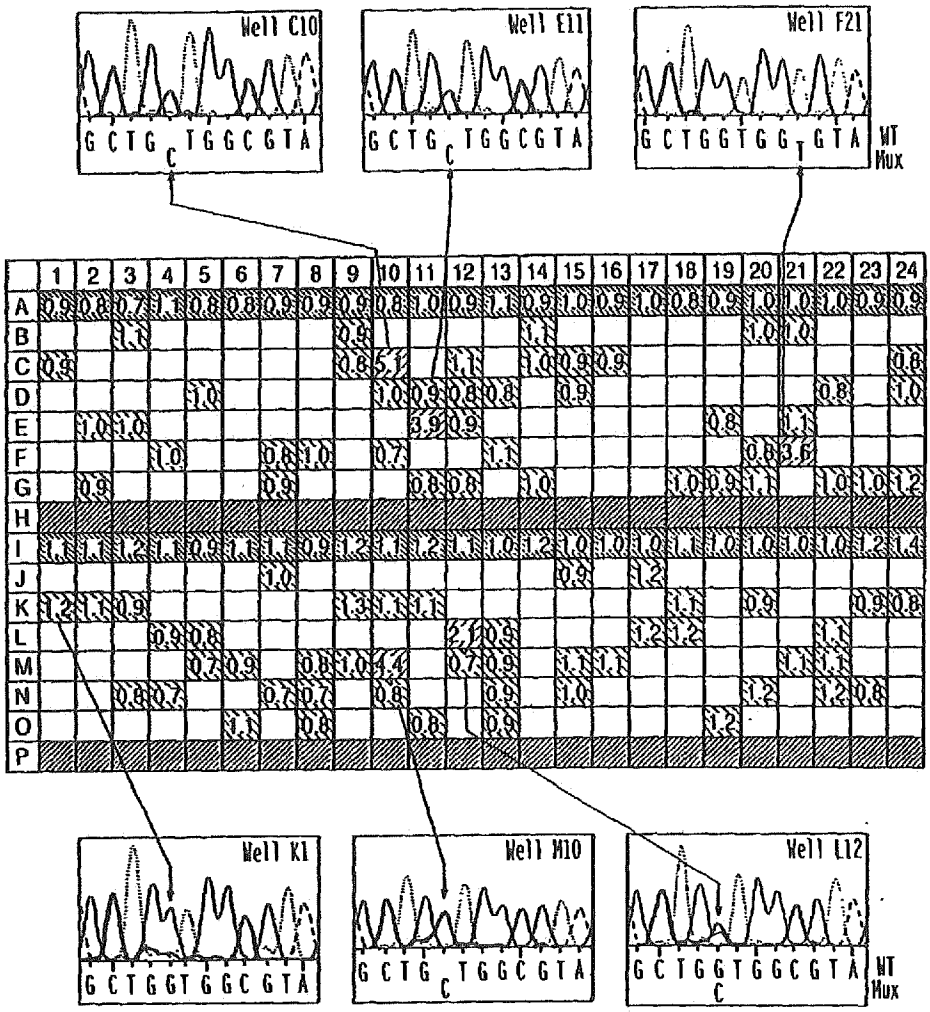
G C T G A T G G C G T A
G C T G A T G G C G T A
WT
Mux
Gly13Asp
4.2
1.0
3.3
0.96
0.85
G C T G G T G A C G T A
G C T G G T G G C G T A
G C T G G T G . C G T A
WT
Mux

FIG. 4

FIG. 5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Well E11
G C T G C T G G C G T A
Well F21
G C T G G T G G T G T A
WT Mux
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A
B
C
D
E
F
G
H
I
J
K
L
M
N
O
P
Well K1
G C T G G T G G C G T A
Well M10
G C T G C T G G C G T A
Well L12
G C T G G T G G C G T A
WT Mux

☒ PATENT ☐ DESIGN B&W Ref. 01107.00021 Date May 21, 2002

☐ HAND CARRY Group/Section _____ Bldg _____ Rm _____

Serial/Patent No. 09/613,826 Atty/Sec SAK/MHS/sel

Inventor Bert Vogelstein et al Client JHU

Title Digital Amplification

MAY 21 2002

The following has been received in the U.S. Patent and Trademark Office on the date stamped hereon:

☐ _____ total pp Spec., including : # of Claims _____
(# of independent claims _____). ☐ Abstract

☒ Drawings : ☒ Formal ☐ Informal 1A, 1B, 1C, 2, 3,
# of distinct sheets 7 : Figs. 4, 5

☐ Declaration/Power of Attorney : ☐ Executed ☐ Unexecuted

☐ Assignment w/PTO Cover Sheet

☐ IDS w/PTO 1449 ☐ References ☐ w/Fee

☐ Preliminary Amendment

☐ Priority Claim (Foreign or U.S. Provisional. B&W # _____)

Country	Appl. #	Date
☐ w/Foreign Priority Document(s)		

☐ Application : ☐ CIP ☐ Continuation ☐ Divisional
Parent Ser. No. _____ B&W# _____

☐ U.S. Provisional _____ pp Spec/Claims; Cover Sheet

☐ Response to Missing Parts/Requirements dtd _____

☐ Response to Notice to File Corrected Appln. Papers dtd _____

☐ Request for Expedited Foreign Filing License

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☐ Response to Restriction/Election Requirement

28

☐ Sequence Listing : ☐ Diskette ☐ Paper

☐ Amendment ☐ Response : OA dtd _____

☐ Petition for Extension of Time until _____

☐ CPA ☐ RCE ☐ w/Ext of Time : OA dtd _____

☐ Request for Approval of Drawing Changes

☐ Notice of Appeal & Fee

☐ Brief : ☐ Appeal & Fee ☐ Reply

☐ Request for Oral Hearing

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Notice of Allowance dtd 2-26-02

☐ Amendment under 37 CFR 1.312

☐ Request for Certificate of Correction

☒ Transmittal ☒ Fee Transmittal w/Auth. to Charge Deposit Acct.

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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	Bert Vogelstein
Group Art Unit	1637
Examiner Name	Jeffrey Siew
Attorney Docket Number	001107.00031

Total Number of Pages In This Submission

ENCLOSURES *(check all that apply)*

- ☐ Fee Transmittal Form
- ☐ Fee Attached
- ☐ Amendment / Response
- ☐ After Final
- ☐ Affidavits/declaration(s)
- ☐ Extension of Time Request
- ☐ Express Abandonment Request
- ☐ Information Disclosure Statement
- ☐ Certified Copy of Priority Document(s)
- ☐ Response to Missing Parts/ Incomplete Application
- ☐ Response to Missing Parts under 37 CFR 1.52 or 1.53
- ☐ Assignment Papers *(for an Application)*
- ☒ Drawing(s) 7 sheets (Figs. 1A, 1B, 1C, 2, 3, 4, and 5)
- ☐ Licensing-related Papers
- ☐ Petition
- ☐ Petition to Convert to a Provisional Application
- ☐ Power of Attorney, Revocation Change of Correspondence Address
- ☐ Terminal Disclaimer
- ☐ Request for Refund
- ☐ CD, Number of CD(s) ____
- ☐ After Allowance Communication to Group
- ☐ Appeal Communication to Board of Appeals and Interferences
- ☐ Appeal Communication to Group *(Appeal Notice, Brief, Reply Brief)*
- ☐ Proprietary Information
- ☒ Submission of Formal Drawings
- ☒ Other Enclosure(s) *(please identify below)*:
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Remarks

SIGNATURE OF APPLICANT, ATTORNEY, OR AGENT

Firm or Individual name	Michelle Holmes-Son, Reg. No. 47,660
Signature	Michelle Holmes-Son
Date	July 8, 2002

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4-25-2002

PATENT

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

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

For: DIGITAL AMPLIFICATION

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Respectfully submitted,

Date: May 21, 2002

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

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Washington, D.C. 20001-4597
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- ☐ **PTO 892**
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- ☐ **PTO 1474**
- ☐ **Assignment**
- ☐ **Cover page**

Additional comments:Paper #25 same as Paper #23

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Fax Cover Sheet

Date: 15 Apr 2002

To: Michelle 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) 508-9100	**Return Fax No.:** 703-308-4556
Re:	**CC:**

☐ **Urgent** ☐ **For Review** ☐ **For Comment** ☐ **For Reply** ☐ **For Your Request**

Comments:
per your request

JSiew

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Fax Cover Sheet

Date: 29 Jun 2001

To: Ms. Holmeson	From: Jeffrey Slew
Application/Control Number: 09/613,528	Art Unit: 1656
Fax No.: (202) 508-9299	Phone No.: 703-305-3998
Voice No.: (202) 508-9100	Return Fax No.: 703-308-4556
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Jeffrey Slew

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Fax No.: (202) 508-9299	**Phone No.:** 703-305-3886
Voice No.: (202) 508-9100	**Return Fax No.:** 703-308-4556
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Jeffrey Siew

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

APPLICATION NUMBER: 08/613826

Total Fee Calculation

	Fee Code	Total # of Claims		Number Extra	X	Fee Ca. Entity	Fee Sm. Entity	=	Total
Basic Filing Fee	101 103						690	-	
Total Claims >20	103 203	64	-20 =	44	X		345	-	
Independent Claims >1	102 202	5	-3 =	2	X		156	-	
Multi. Dep Claim Present	104 204						130	-	
Surcharge	105 205							-	
English Translation	139								
TOTAL FEE CALCULATION									1768

Fees due upon filing the application is:

Total Filing Fees Due = $ 1768

Less Filing Fees Submitted - $ /

BALANCE DUE = $ 1768

Office of Initial Patent Examination

Figure 7

FORM OPE-RAM-01 (Rev. 12/97)

PATENT COOPERATION TREATY

From the INTERNATIONAL SEARCHING AUTHORITY 01107.00030

PCT

NOTIFICATION OF TRANSMITTAL OF THE INTERNATIONAL SEARCH REPORT OR THE DECLARATION

(PCT Rule 44.1)

To:
BANNER & WITCOFF, LTD.
Attn. KAGAN, Sarah A.
1001 G Street, N.W.
Eleventh Floor
Washington, DC 20001-4597
UNITED STATES OF AMERICA

DOCKETED
JAN 2
Article 19 Amendment
21 FE 2002

	Date of mailing (day/month/year) 21/12/2001
Applicant's or agent's file reference 01107.00030	FOR FURTHER ACTION See paragraphs 1 and 4 below
International application No. PCT/US 00/20740	International filing date (day/month/year) 31/07/2000
Applicant THE JOHNS HOPKINS UNIVERSITY et al.	

1. [X] 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 90*bis*.1 and 90*bis*.3, respectively, before the completion of the technical preparations for international publication.

Within 19 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.

Name and mailing address of the International Searching Authority	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL-2280 HV Rijswijk Tel. (+31-70) 340-2040, Tx. 31 651 epo nl, Fax: (+31-70) 340-3016	Catherine Humbert

Form PCT/ISA/220 (July 1998)

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 latter 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 cancelled, 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 205(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 46.4)

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).

Consequence 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	FOR FURTHER ACTION	see Notification of Transmittal of International Search Report (Form PCT/ISA/220) as well as, where applicable, item 5 below.
01107.00030		
International application No.	International filing date *(day/month/year)*	(Earliest) Priority Date *(day/month/year)*
PCT/US 00/20740	31/07/2000	02/08/1999
Applicant		
THE JOHNS HOPKINS UNIVERSITY et al.		

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.

[X] 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 readble 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 II).

4. With regard to the title,

   [X] 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,

   [X] 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.

   [X] None of the figures.

Form PCT/ISA/210 (first sheet) (July 1998)

INTERNATIONAL SEARCH REPORT

International Application No
PCT/US 00/20740

A. CLASSIFICATION OF SUBJECT MATTER
IPC 7 C12Q1/68

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

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
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)
EPO-Internal, WPI Data, PAJ, BIOSIS, CHEM ABS Data, EMBASE, MEDLINE, SCISEARCH

C. DOCUMENTS CONSIDERED TO BE RELEVANT

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." 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 May 25, 1999 ISSN: 0027-8424 the whole document --- -/--	1-13, 15-19, 21-24, 30,32, 38-45, 47-51, 53-56, 62,64

[X] Further documents are listed in the continuation of box C.

[X] 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

"E" earlier document but published on or after the international filing date

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

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

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

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

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

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

"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
10 December 2001	21/12/2001

Name and mailing address of the ISA	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+31-70) 340-2040, Tx 31 651 epo nl, Fax: (+31-70) 340-3016	Gabriels, J

Form PCT/ISA/210 (second sheet) (July 1992)

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.
X	PIATEK AMY S ET AL: "Molecular beacon sequence analysis for detecting drug resistance in Mycobacterium tuberculosis." NATURE BIOTECHNOLOGY, vol. 16, no. 4, April 1998 (1998-04), pages 359-363, XP000891876 ISSN: 1087-0156 the whole document	1-13, 15-19, 21-24, 26,27, 30,32, 38-45, 47-51, 53-56, 58,59, 62,64
X	TYAGI SANJAY ET AL: "Multicolor molecular beacons for allele discrimination." NATURE BIOTECHNOLOGY, vol. 16, no. 1, January 1998 (1998-01), pages 49-53, XP002143901 ISSN: 1087-0156 the whole document	1-13, 15-19, 21-24, 26,27, 30,32, 38-45, 47-51, 53-56, 58,59, 62,64
X	TYAGI SANJAY ET AL: "Molecular beacons: Probes that fluoresce upon hybridization." NATURE BIOTECHNOLOGY, vol. 14, no. 3, 1996, pages 303-308, XP002914999 ISSN: 1087-0156 the whole document	1-19, 21-23, 30, 38-51, 53-55,62
X	WO 99 13113 A (KRAMER FRED R ;ALLAND DAVID (US); PIATEK AMY (US); TYAGI SANJAY (U) 18 March 1999 (1999-03-18) page 7, line 32 -page 9, line 3; claims 1-14; example 2	1-19, 21-23, 30, 38-51, 53-55,62
X	HALFORD WILLIAM P ET AL: "The inherent quantitative capacity of the reverse transcription-polymerase chain reaction." ANALYTICAL BIOCHEMISTRY, vol. 266, no. 2, 15 January 1999 (1999-01-15), pages 181-191, XP000791527 ISSN: 0003-2697 the whole document	1-12, 38-44
X	EP 0 643 140 A (CANON KK) 15 March 1995 (1995-03-15) page 1-3	1-12, 38-44

-/--

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Form PCT/ISA/210 (continuation of second sheet) (July 1992)

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.
X	WO 95 13399 A (NEW YORK HEALTH RES INST) 18 May 1995 (1995-05-18) claims 1,2	33-37
A	US 5 804 383 A (GRUENERT DIETER C ET AL) 8 September 1998 (1998-09-08) claim 1	25,28, 57,60
A	US 5 858 663 A (NISSON PAUL E ET AL) 12 January 1999 (1999-01-12) claim 1	25,28, 57,60
A	DATABASE BIOSIS 'Online! BIOSCIENCES INFORMATION SERVICE, PHILADELPHIA, PA, US; April 1999 (1999-04) EVERETT KARIN D E ET AL: "Identification of nine species of the Chlamydiaceae using PCR-RFLP." Database accession no. PREV199900271658 XP002185145 abstract & INTERNATIONAL JOURNAL OF SYSTEMATIC BACTERIOLOGY, vol. 49, no. 2, April 1999 (1999-04), pages 803-813, ISSN: 0020-7713	20,52
P,X	VOGELSTEIN BERT ET AL: "Digital PCR." PROCEEDINGS OF THE NATIONAL ACADEMY OF SCIENCES OF THE UNITED STATES, vol. 96, no. 16, 3 August 1999 (1999-08-03), pages 9236-9241, XP002185144 Aug. 3, 1999 ISSN: 0027-8424 the whole document	1-64

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Form PCT/ISA/210 (continuation of second sheet) (July 1992)

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
WO 9913113 A	18-03-1999	AU	9484698 A	29-03-1999
		EP	1012344 A1	28-06-2000
		JP	2001515734 T	25-09-2001
		WO	9913113 A1	18-03-1999
EP 0643140 A	15-03-1995	JP	7163399 A	27-06-1995
		AT	207966 T	15-11-2001
		CA	2126391 A1	14-03-1995
		DE	69428855 D1	06-12-2001
		EP	0643140 A1	15-03-1995
		US	5670315 A	23-09-1997
		AT	206210 T	15-10-2001
		DE	69330818 D1	31-10-2001
		EP	1052291 A2	15-11-2000
		EP	0603783 A1	29-06-1994
		JP	9040661 A	10-02-1997
		JP	7174759 A	14-07-1995
		SG	65577 A1	22-06-1999
		US	5624798 A	29-04-1997
		US	6022961 A	08-02-2000
WO 9513399 A	18-05-1995	AU	697841 B2	15-10-1998
		AU	1292195 A	29-05-1995
		CA	2176348 A1	18-05-1995
		EP	0728218 A1	28-08-1996
		JP	9504950 T	20-05-1997
		WO	9513399 A1	18-05-1995
		US	6103476 A	15-08-2000
		US	5925517 A	20-07-1999
US 5804383 A	08-09-1998	US	6010908 A	04-01-2000
		AU	5087193 A	15-03-1994
		EP	0656747 A1	14-06-1995
		WO	9404032 A1	03-03-1994
		AU	7262696 A	30-04-1997
		WO	9713869 A1	17-04-1997
US 5858663 A	12-01-1999	US	5547838 A	20-08-1996

PATENT APPLICATION FEE DETERMINATION RECORD
Effective December 29, 1999

Application or Docket Number

CLAIMS AS FILED - PART I

FOR	(Column 1) NUMBER FILED		(Column 2) NUMBER EXTRA
BASIC FEE			
TOTAL CLAIMS	64	minus 20=	* 44
INDEPENDENT CLAIMS	5	minus 3 =	* 2
MULTIPLE DEPENDENT CLAIM PRESENT			

* If the difference in column 1 is less than zero, enter "0" in column 2

SMALL ENTITY TYPE ☐		OR	OTHER THAN SMALL ENTITY	
RATE	FEE		RATE	FEE
	345.00	OR		690.00
X$ 9=		OR	X$18=	792
X39=		OR	X78=	156
+130=		OR	+260=	
TOTAL		OR	TOTAL	948

CLAIMS AS AMENDED - PART II

AMENDMENT A

	(Column 1) CLAIMS REMAINING AFTER AMENDMENT		(Column 2) HIGHEST NUMBER PREVIOUSLY PAID FOR	(Column 3) PRESENT EXTRA
Total	*	Minus	**	=
Independent	*	Minus	***	=
FIRST PRESENTATION OF MULTIPLE DEPENDENT CLAIM				

SMALL ENTITY		OR	OTHER THAN SMALL ENTITY	
RATE	ADDITIONAL FEE		RATE	ADDITIONAL FEE
X$ 9=		OR	X$18=	
X39=		OR	X78=	
+130=		OR	+260=	
TOTAL ADDIT. FEE		OR	TOTAL ADDIT. FEE	

AMENDMENT B

	(Column 1) CLAIMS REMAINING AFTER AMENDMENT		(Column 2) HIGHEST NUMBER PREVIOUSLY PAID FOR	(Column 3) PRESENT EXTRA
Total	*	Minus	**	=
Independent	*	Minus	***	=
FIRST PRESENTATION OF MULTIPLE DEPENDENT CLAIM				

RATE	ADDITIONAL FEE		RATE	ADDITIONAL FEE
X$ 9=		OR	X$18=	
X39=		OR	X78=	
+130=		OR	+260=	
TOTAL ADDIT. FEE		OR	TOTAL ADDIT. FEE	

AMENDMENT C

	(Column 1) CLAIMS REMAINING AFTER AMENDMENT		(Column 2) HIGHEST NUMBER PREVIOUSLY PAID FOR	(Column 3) PRESENT EXTRA
Total	*	Minus	**	=
Independent	*	Minus	***	=
FIRST PRESENTATION OF MULTIPLE DEPENDENT CLAIM				

RATE	ADDITIONAL FEE		RATE	ADDITIONAL FEE
X$ 9=		OR	X$18=	
X39=		OR	X78=	
+130=		OR	+260=	
TOTAL ADDIT. FEE		OR	TOTAL ADDIT. 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.

EXHIBIT 5

US005928870A

United States Patent [19]

Lapidus et al.

[11] **Patent Number: 5,928,870**

[45] **Date of Patent: *Jul. 27, 1999**

[54] **METHODS FOR THE DETECTION OF LOSS OF HETEROZYGOSITY**

[75] Inventors: **Stanley N. Lapidus**, Bedford, N.H.; **Anthony P. Shuber**, Milford, Mass.

[73] Assignee: **Exact Laboratories, Inc.**, Maynard, Mass.

[*] Notice: This patent is subject to a terminal disclaimer.

[21] Appl. No.: **08/876,857**

[22] Filed: **Jun. 16, 1997**

[51] **Int. Cl.**[6] **C12Q 1/68**

[52] **U.S. Cl.** **435/6**; 536/24.3

[58] **Field of Search** 435/6; 536/24.3

[56] **References Cited**

U.S. PATENT DOCUMENTS

4,101,279	7/1978	Aslam .
4,309,782	1/1982	Paulin .
4,333,734	6/1982	Fleisher .
4,445,235	5/1984	Slover et al. .
4,535,058	8/1985	Weinberg et al. .
4,683,195	7/1987	Mullis et al. .
4,705,050	11/1987	Markham .
4,735,905	4/1988	Parker .
4,786,718	11/1988	Weinberg et al. .
4,857,300	8/1989	Maksem .
4,871,838	10/1989	Bos et al. .
4,981,783	1/1991	Augenlicht .
4,982,615	1/1991	Sultan et al. .
5,087,617	2/1992	Smith .
5,126,239	6/1992	Livak et al. .
5,137,806	8/1992	LeMaistre et al. .
5,149,506	9/1992	Skiba et al. .
5,196,167	3/1993	Guadagno et al. .
5,248,671	9/1993	Smith .
5,272,057	12/1993	Smulson et al. .
5,302,509	4/1994	Cheeseman .
5,330,892	7/1994	Vogelstein et al. .
5,331,973	7/1994	Fiedler et al. .
5,348,855	9/1994	Dattagupta et al. .
5,352,775	10/1994	Albertsen et al. .
5,362,623	11/1994	Vogelstein et al. .
5,369,004	11/1994	Polymeropoulos et al. .
5,378,602	1/1995	Polymeropoulos et al. .
5,380,645	1/1995	Vogelstein .
5,380,647	1/1995	Bahar .
5,382,510	1/1995	Levine et al. .
5,409,586	4/1995	Kamahori et al. .
5,458,761	10/1995	Kamahori et al. .
5,463,782	11/1995	Carlson et al. .
5,466,576	11/1995	Schulz et al. .
5,468,610	11/1995	Polymeropoulos et al. .
5,468,613	11/1995	Erlich et al. .
5,489,508	2/1996	West et al. .
5,492,808	2/1996	de la Chapelle et al. .
5,496,470	3/1996	Lenhart .
5,508,164	4/1996	Kausch et al. .
5,512,441	4/1996	Ronal .
5,514,547	5/1996	Balazs et al. .
5,527,676	6/1996	Vogelstein et al. .
5,532,108	7/1996	Vogelstein .
5,580,729	12/1996	Vogelstein .
5,709,998	1/1998	Kinzler et al. .

FOREIGN PATENT DOCUMENTS

A-11325/95	10/1994	Australia .
0 284 362 A2	9/1988	European Pat. Off. .
0 337 498	10/1989	European Pat. Off. .
0 390 323 A2	10/1990	European Pat. Off. .
0 390 323 A3	10/1990	European Pat. Off. .
0 407 789 A1	1/1991	European Pat. Off. .
0 407 789 B1	1/1991	European Pat. Off. .
0 608 004 A2	7/1994	European Pat. Off. .
0 259 031 B1	11/1994	European Pat. Off. .
0 664 339 A1	7/1995	European Pat. Off. .
WO 92/13103	8/1992	WIPO .
WO 93/18186	9/1993	WIPO .
WO 93/20233	10/1993	WIPO .
WO 94/00603	1/1994	WIPO .
WO 94/09161	4/1994	WIPO .
WO 94/10575	5/1994	WIPO .
WO 94/11383	5/1994	WIPO .
WO 95/07361	3/1995	WIPO .
WO 95/09928	4/1995	WIPO .
WO 95/09929	4/1995	WIPO .
WO 95/12606	5/1995	WIPO .
WO 95/13397	5/1995	WIPO .
WO 95/15400	6/1995	WIPO .
WP 95/16792	6/1995	WIPO .
WO 95/18818	7/1995	WIPO .
WO 95/19448	7/1995	WIPO .
WO 95/25813	9/1995	WIPO .
WO 95/31728	11/1995	WIPO .
WO 96/01907	1/1996	WIPO .
WO 96/06951	3/1996	WIPO .
WO 96/08514	3/1996	WIPO .
WO 96/12821	5/1996	WIPO .
WO 96/13611	5/1996	WIPO .

OTHER PUBLICATIONS

Bos et al., Nature 327:293–297, May 1987.

Segel, "Biochemical Calculations," John Wiley & Sons, Inc. NY, 1976.

Sanger, F., S. Nicklen and A.R. Coulson (Dec. 1977) "DNA sequencing with chain–terminating inhibitors" vol. 74, No. 12 *Proc. Natl. Acad. Sci. USA* pp. 5463–5467.

Wallace R.B., et al. (1979) "Hybridization of synthetic oligodeoxyribonucleotides to Φ_{102} 174 DNA: the effect of single base pair mismatch" vol. 6, No. 11 *Nucleic Acids Research* pp. 3543–3557.

Coll P., K. Phillips, and F. C. Tenover (Oct. 1989) "Evaluation of a Rapid Method of Extracting DNA from Stool Samples for Use in Hybridization Assays" vol. 27, No. 10 *Journal of Clinical Microbiology* pp. 2245–2248.

Jessup J. M. and G. E. Gallick (Sep./Oct. 1992) "The Biology of Colorectal Carcinoma" *Current Problems in Cancer* pp. 263–328.

(List continued on next page.)

Primary Examiner—Scott W. Houtteman

Attorney, Agent, or Firm—Testa, Hurwitz & Thibeault,LLP

[57] **ABSTRACT**

Methods are provided for detecting loss of heterozygosity in a nucleic acid sample. These methods are particularly useful for identifying individuals with gene mutations indicative of early colorectal cancer.

25 Claims, 3 Drawing Sheets

OTHER PUBLICATIONS

Litia A., L. Liukkonen and H. Siitari (1992) "Simultaneous detection of two cystic fibrosis alleles using dual–label time–resolved fluorometry" 6 *Molecular and Cellular Probes* pp. 505–512.

Young G.P., and B.H. Demediu (1992) "The genetics, epidemiology, and early detection of gastrointestinal cancers" 4 *Current Opinion in Oncology* pp. 728–735.

Hoss M., et al. (Sep. 17, 1992) "Excrement analysis by PCR" *Scientific Correspondence* p. 199.

Sidransky, et al. (Apr. 3, 1992) "Identification of ras Oncogene Mutations in the Stool of Patients with Curable Colorectal Tumors" vol. 256 *Science* pp. 102–105.

Takeda S., S. Ichii, and Y. Nakamura (1993) "Detection of K–ras Mutation in Sputum by Mutant–Allele–Specific Amplification (MASA)" 2 *Human Mutation* pp. 112–117.

Leong P.K., et al. (1993) "Detection of MYCN Gene Amplification and Deletions of Chromosome 1p in Neuroblastoma by In Situ Hybridization Using Routine Histologic Sections" vol. 69, No. 1 *Laboratory Investigations* pp. 43–50.

Thibodeau S.N., G. Bren, D. Schaid (May 7, 1993) "Microsatellite Instability in Cancer of the Proximal Colon" vol. 260 *Science* pp. 816–819.

Naber S. P.(Dec. 1, 1994) "Molecular Pathology—Detection of Neoplasia" 331 *New England Journal of Medicine* pp. 1508–1510.

Cave H., et al. (1994) "Reliability of PCR Directly from Stool Samples: Usefulness of an Internal Standard" vol. 16, No. 5 *BioTechniques* pp. 809–810.

Caldas, C., et al (Jul. 1, 1994) "Detection of K–ras Mutations in the Stool of Patients with Pancreatic Adenocarcinoma and Pancreatic Ductal Hyperplasia" 54 *Cancer Research* pp. 3568–3573.

Charlesworth B., P. Sniegowski and W. Stephan (Sep. 15, 1994) "The evolutionary dynamics of repetitive DNA in eukaryotes" vol. 371 *Nature* pp. 215–220.

Fearon E. R.(1995) "16 Molecular Abnormalities in Colon and Rectal Cancer" *The Molecular Basis of Cancer* pp. 340–357.

Ravelingien N., J. C. Pector & T. Velu (1995) "Contribution of molecular oncology in the detection of colorectal carcinomas" 58 *Acta Gastro–Enterologica Belgica* pp. 270–273.

Duffy M.J.(1995) "Can Molecular Markers Now Be Used for Early Diagnosis of Malignancy?" 41/10*Clin. Chem.* pp. 1410–1413.

Blum H.E.(1995) "Colorectal Cancer: Future Population Screening for Early Colorectal Cancer" vol. 31A *European Journal of Cancer,* pp. 1369–1372.

Ridanpaa M., S. Anttila and K. Husgafvel–Pursiainen (1995) "Detection of Loss of Heterozygosity in the p53 Tumor Suppressor Gene Using a PCR–based Assay" 191 *Path. Res. Pract.* pp. 399–402.

Smith–Ravin J., J. England, I.C. Talbot, W. Bodmer (1995) "Detection of c–Ki–ras mutations in faecal samples from sporadic colorectal cancer patients" 36 *Gut* pp. 81–86.

Orlow I., et al. (Oct. 18, 1995) "Deletion of the p16 and p15 Genes in Human Bladder Tumors" vol. 87, No. 20 *Journal of the National Cancer Institute* pp. 1524–1529.

Hasegawa, Y., et al., (1995) "Detection of K–ras mutations in DNAs isolated from feces of patients with colorectal tumors by mutant–allele–specific amplification (MASA)" 10 *Oncogene* pp. 1441–1445.

Loktionov A. and I. K. O'Neill (1995) "Early detection of cancer–associated gene alterations in DNA isolated from rat feces during intestinal tumor induction with 1,2–dimethylhydrazine" 6 *International Journal of Oncology* pp. 437–445.

Honchel R., K. C. Halling and S. N. Thibodeau (1995) "Genomic instability in neoplasia" vol. 6 *Seminars in Cell Biology* pp. 45–52.

Deuter R., S. Pietsch, S. Hertel and O. Muller (1995) "A method for preparation of fecal DNA suitable for PCR" vol. 23, No. 18 *Nucleic Acids Research* pp. 3800–3801.

Dib C., et al. (Mar. 14, 1996) "A comprehensive genetic map of the human genome based on 5,264 microsatellites" vol. 380 *Nature* pp. 152–154.

Cunningham C. and M.G. Dunlop (1996) "Molecular genetic basis of colorectal cancer susceptibility" 83 *British Journal of Surgery* pp. 321–329.

Mao L., et al. (Feb. 2, 1996) "Molecular Detection of Primary Bladder Cancer by Microsatellite Analysis" vol. 271 *Science* pp. 659–662.

Villa E., et al. (May 1996) "Identification of Subjects at Risk for Colorectal Carcinoma Through a Test Based on K–ras Determination in the Stool" vol. 110, No. 5 *Gastroenterology* pp. 1346–1353.

Nollau P., C. Moser, G. Weinland, and C. Wagener (1996) "Detection of K–ras Mutations in Stools of Patients with Colorectal Cancer by Mutant–enriched PCR" 66 *Int. J. Cancer* pp. 332–336.

Eguchi S., N. Kohara, K. Komuta, and T. Kanematsu (Apr. 15, 1996) "Mutations of the p53 Gene in the Stool of Patients with Resectable Colorectal Cancer" vol. 77, No. 8 *Cancer Supplement* pp. 1707–1710.

Nollau P., C. Moser, and C. Wagener (May 1996) "Isolation of DNA from Stool and Bodily Fluids for PCR Amplication" vol. 20, No. 5 *BioTechniques* pp. 784–788.

Rhyu M. S. (Mar. 6, 1996) "Molecular Mechanisms Underlying Hereditary Nonpolyposis Colorectal Carcinoma" vol. 88, No. 5 *Journal of the National Cancer Institute* pp. 240–251.

Gyllensten U. B., Allen M. (1995) "Sequencing of In Vitro Amplified DNA" In *Recombinant DNA Methodology II* (Wu, ed) pp. 565–578.

Myers, R.M., "The Pulses of Subtraction", vol. 259 Science, pp. 942–943 (1993).

Jonsson et al., From Mutation mapping to phenotype cloning: vol. 92 Proc. Natl. Sci. USA, pp. 83–85 (1995).

Watson et al. "Isolation of Differentiality Expressed Sequence Tags from Human Breast Cancer" Advanced in Brief XP 000576043, pp. 4598–4602.

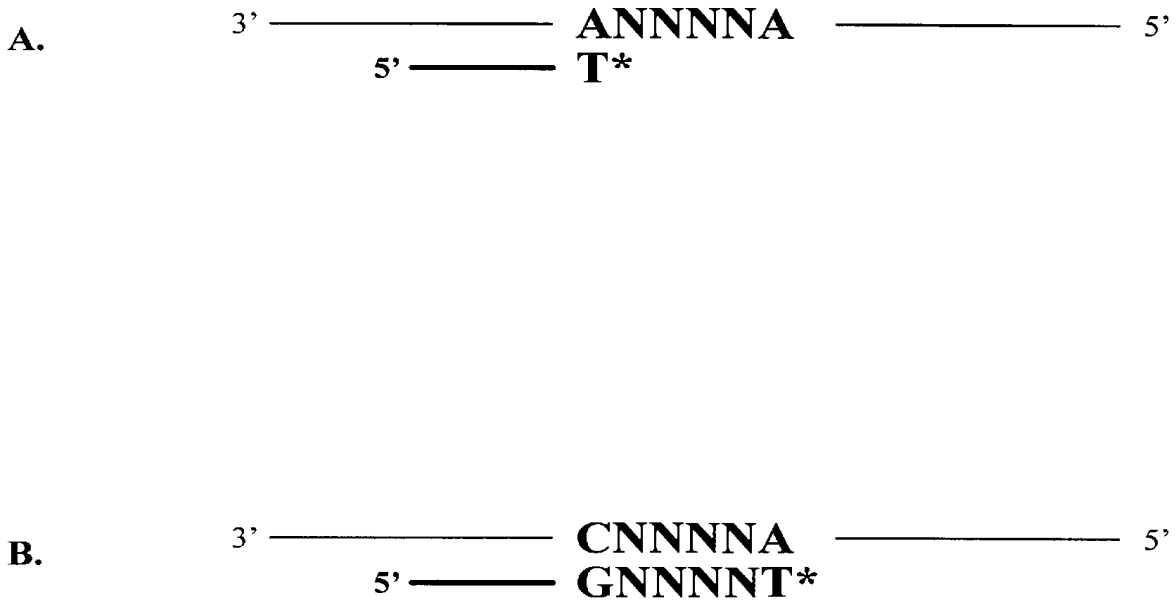


FIG. 1

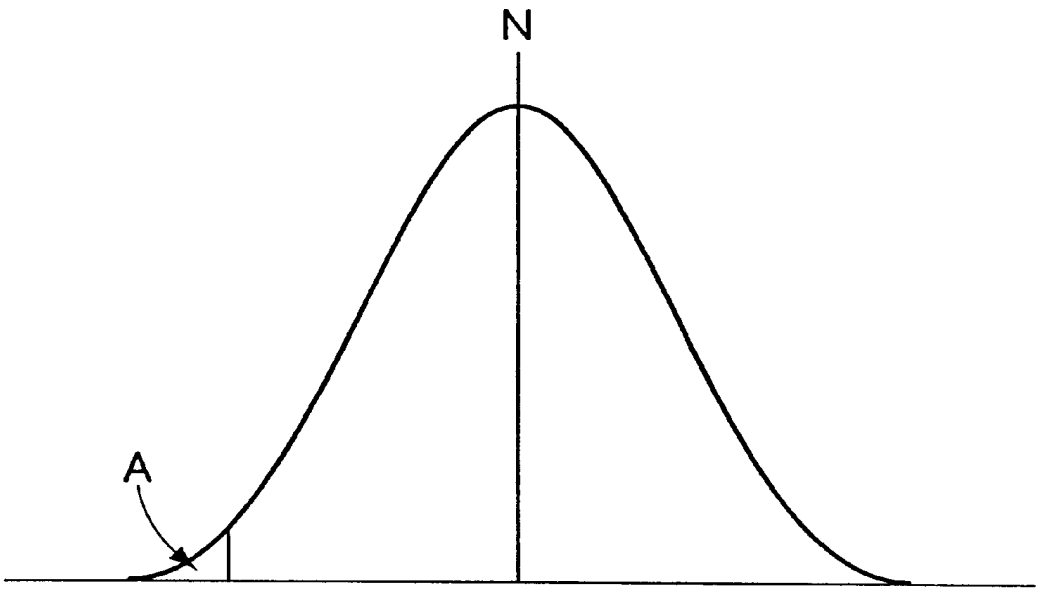


FIG. 2A

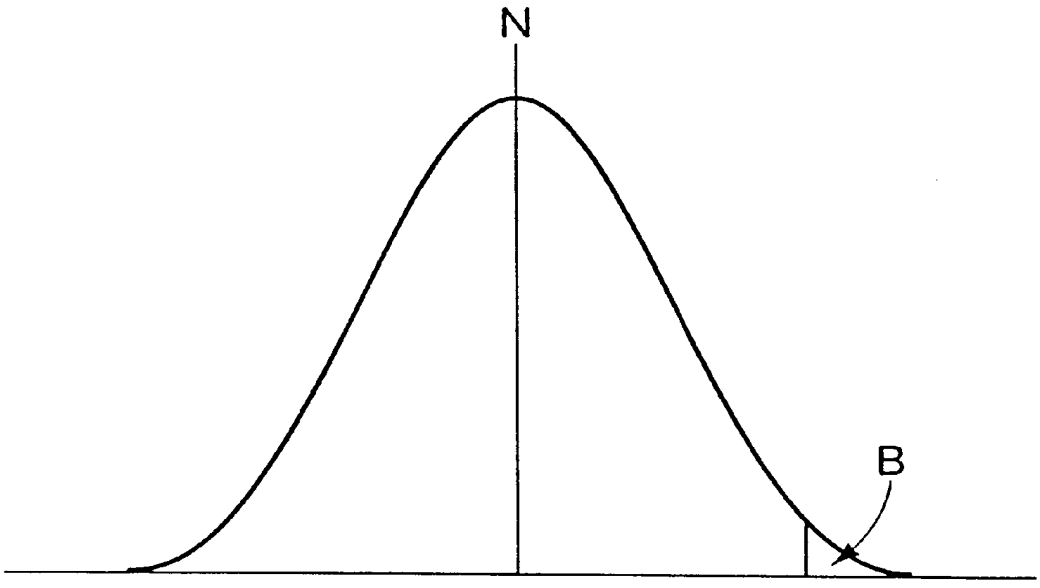


FIG. 2B

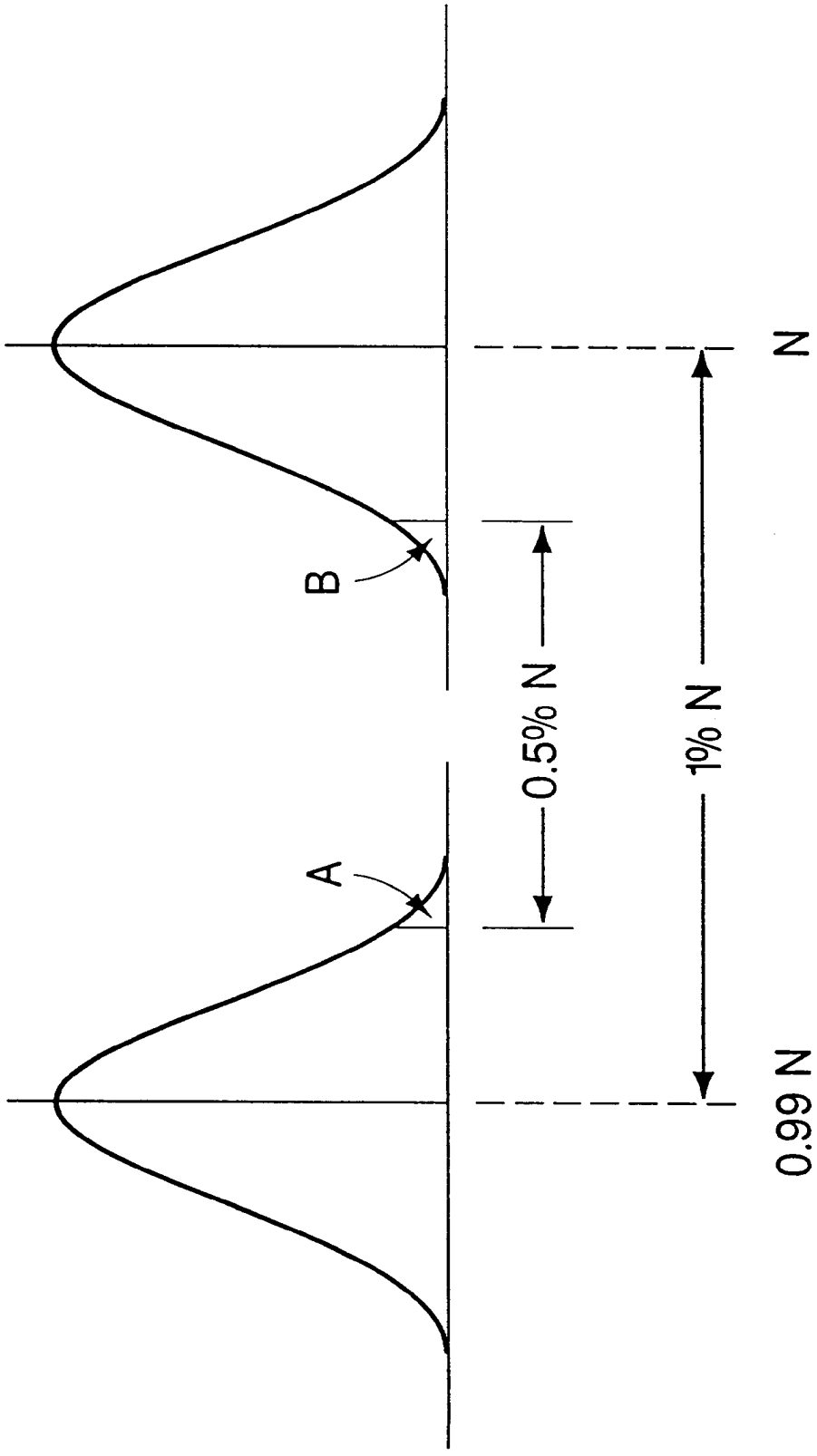


FIG. 3

METHODS FOR THE DETECTION OF LOSS OF HETEROZYGOSITY

FIELD OF THE INVENTION

This invention relates to methods useful for disease diagnosis by detecting loss of heterozygosity in cellular samples containing a small amount of mutated genetic material dispersed within a large amount of normal genetic material. Methods of the invention are especially useful in the detection of genetic mutations characteristic of cancer.

BACKGROUND OF THE INVENTION

Cancer is a disease characterized by genomic instability. The acquisition of genomic instability is thought to arise from a coincident disruption of genomic integrity and a loss of cell cycle control mechanisms. Generally, a disruption of genomic integrity is thought merely to increase the probability that a cell will engage in the multistep pathway leading to cancer. However, coupled with a loss of cell cycle control mechanisms, a disruption in genomic integrity may be sufficient to generate a population of genomically unstable neoplastic cells. A common genetic change characteristic of the early stages of transformation is loss of heterozygosity. Loss of heterozygosity at a number of tumor suppressor genes has been implicated in tumorigenesis. For example, loss of heterozygosity at the P53 tumor suppressor locus has been correlated with various types of cancer. Ridanpaa, et al., *Path. Res. Pract,* 191: 399–402 (1995). The loss of the apc and dcc tumor suppressor genes has also been associated with tumor development. *Blum, Europ. J. Cancer,* 31A: 1369–372 (1995).

Loss of heterozygosity is therefore a potentially useful marker for detecting the early stages of cancer. However, in the early stages of cancer only a small number of cells within a tissue have undergone transformation. Genetic changes characteristic of genomic instability theoretically can serve as markers for the early stages of, for example, colon cancer, and can be detected in DNA isolated from biopsied colonic epithelium and in some cases from transformed cells shed into fecal material. Sidransky, et al., *Science,* 256: 102–105 (1992).

Detection methods proposed in the art are time-consuming and expensive. Duffy, supra. Moreover, methods according to the art cannot be used to identify a loss of heterozygosity or microsatellite instability in small subpopulation of cells when the cells exist in a heterogeneous (i.e., clonally impure) sample. For example, in U.S. Pat. No. 5,527,676, it is stated that tissue samples in which a mutation is to be detected should be enriched for tumor cells in order to detect the loss of heterozygosity in a p53 gene.

Colorectal cancer is a common cause of death in Western society. Any tumor or precancerous polyp that develops along the length of the colon or the rectum sheds cells or DNA into the lumen of the colon. Shed cells or cellular DNA are usually incorporated onto and into stool as stool passes through the colon. In the early stages of cancer, cancerous or precancerous cells represent a very small fraction of the shed epithelial cells or DNA in stool. Current methods for detection of colorectal cancer do not focus on detecting cancerous or precancerous cells in stool. Rather, such methods typically focus on extracellular indicia of the presence of cancer, such as the presence of fecal occult blood or carcinoembryonic antigen circulating in serum.

It is thought that sporadic colorectal cancers result from mutations in oncogenes and tumor suppressor genes. Sporadic colorectal cancer is also typically associated with

massive loss of genetic material. Such mutations appear to occur at a point in the etiology of the disease that is much earlier than the point at which extracellular indicia or clinical signs of cancer are observed. If detected early, colon cancer may be effectively treated by surgical removal of the cancerous tissue. Surgical removal of early-stage colon cancer is usually successful because colon cancer begins in cells of the colonic epithelium and is isolated from the general circulation until the occurrence of invasion through the epithelial lining. Thus, detection of early mutations in colorectal cells would greatly increase survival rate.

Current non-invasive methods for detection of colon cancer involve the detection of fecal occult blood and carcinoembryonic antigen. These methods often either fail to detect colorectal cancer or they detect colorectal cancer only after it has progressed to a less treatable stage. Moreover, carcinoembryonic antigen is thought not to be an effective predictor of cancer but merely an indicator of recurrent cancer.

Invasive techniques, such as endoscopy, while effective, are expensive and painful and suffer from low patient compliance. Accordingly, current colon cancer screening methods are not practical for screening large segments of the population. See, e.g., *Blum, Europ. J. Cancer,* 31A: 1369–1372 (1995).

Therefore, there is a need in the art for simple and efficient non-invasive methods for reliable large-scale screening to identify individuals with early stage colon cancer. Such methods are provided herein.

SUMMARY OF THE INVENTION

The present invention provides methods for detecting a subpopulation of genomically transformed cells or cellular debris. Such methods detect the presence in a biological sample of a clonal subpopulation of cells which have a genome different from that of the wild type, and from bacterial, parasitic, or contaminating organisms that may also be present in the sample. Practice of the invention permits, for example, detection of a trace amount of DNA derived from cancer or precancer cells in a biological sample containing a majority of "normal" DNA. A preferred use of the methods is to reliably detect in a stool sample voided by a patient the presence of a trace amount of cells and/or cellular debris containing DNA shed into the colon at the site of an asymptomatic precancerous or cancerous lesion. The invention takes advantage of several important insights which permit, for example, reliable detection of a DNA deletion at a known genomic site characteristic of a known cancer cell type. Methods of the invention are useful for the detection and diagnosis of a genetic abnormality, such as a loss of heterozygosity or, more generally, a mutation, which can be correlated with a disease, such as cancer. For purposes of the present invention, unless the context requires otherwise, a "mutation" includes modifications, rearrangements, deletions, substitutions, and additions in a portion of genomic DNA or its corresponding mRNA.

In general, the invention comprises methods for counting (i.e. enumerating) the number of molecules of a target genomic sequence present in a sample. The invention further comprises methods for comparing the number of molecules with a reference number to determine whether any difference between the two numbers is statistically significant, a statistically significant difference being indicative of loss of heterozygosity involving a genomic region comprising the target sequence. A useful reference number is the number of molecules of a reference genomic sequence. The reference

genomic sequence is chosen such that the numbers of molecules of the target and reference genomic sequences are identical in normal cells which have not undergone loss of heterozygosity. When comparing the quantities of two genomic sequences in a sample, the enumerative methods are useful to identify a statistically-significant difference between the two quantities, and to correlate any difference, to a degree of defined statistical confidence, with the presence in the sample of a subpopulation of cells having an altered (e.g. Loss of heterozygosity) genomic sequence.

The invention may be divided into two general embodiments. (1) In a first general embodiment, an enumerative amount (number of copies) of a genetic region of interest in a sample (i.e. including a gene or genes, the mutation of which is known or suspected to be associated with cancer) is compared to an enumerative amount of a reference gene or gene fragment in the sample, the reference gene being a gene which is not normally associated with cancer and which normally has a low rate of mutation. A statistically-significant difference between the two enumerative amounts is indicative of genomic instability in a cellular subpopulation in the sample. (2) In a second general embodiment of the invention, an enumerative amount of a region on a maternal allele is compared to an enumerative amount of the corresponding region on a paternal allele. A statistically-significant difference between the two amounts is indicative of genomic instability.

In a preferred embodiment, enumerative detection of a nucleic acid mutation is accomplished by exposing a nucleic acid sample to first and second radionucleotides. The radionucleotides may be single nucleotides or oligonucleotide probes. The first radionucleotide is capable of hybridizing to a genetic region suspected to be mutated in cancer or precancer cells. The second radionucleotide is capable of hybridizing to a region known not to be mutated in cancer or precancer cells. After washing to remove unhybridized radionucleotides, the number of each of first and second radionucleotides is counted. A statistically-significant difference between the number of first and second radionucleotides is indicative of a mutation in a subpopulation of nucleic acids in the sample.

In preferred methods of the invention, first and second radionucleotides are isolated from other sample components by, for example, gel electrophoresis, chromatography, and mass spectrometry. Also in a preferred embodiment, either or both of the first and second radionucleotides is a chain terminator nucleotide, such as a dideoxy nucleotide. A preferred radionucleotide for use in methods of the invention is selected from the group consisting of ^{32}P, ^{33}P, ^{35}S, ^{3}H, ^{125}I, and ^{14}C. The number of first and second radionucleotides may be determined by counting. Methods of the invention are especially useful for the detection of massive nucleotide deletions, such as those that occur in loss of heterozygosity.

A massive loss of genetic material is detected as a reduction in the expected number in a sample of a nucleic acid fragment that is chosen to represent a genomic region suspected to be lost. For example, deletion of regions including all or part of human chromosome 18q have been associated with the development of cancer. According to the invention, a reduction in the number of cells in a sample having an intact 18q region is determined by comparing the number of a portion of the 18q region detected in the sample to the number of that region expected to occur in the sample. Similarly, a point mutation is detected by methods of the invention as a reduction in the sample of the number of wild-type nucleic acids encompassing the nucleotide suspected to be mutated. Accordingly, methods of the invention detect a mutation by detecting a reduction in the number of a nucleic acid expected to be in a sample. As described in detail below, methods of the invention are useful to detect a mutation in a heterogeneous cellular population without requiring the detection of multiple mutations.

An additional feature of the invention is that it has now been recognized that materials from cells lining the colon (e.g., a polyp or lesion) are shed onto forming stool only in a region comprising a longitudinal stripe along the length of the stool. Thus, unless the stool sample under investigation is a whole stool or comprises at least a cross-section of a stool, the sample will contain the relevant diagnostic information only by chance. The colon contains numerous bends and folds throughout its length. See, U.S. patent application Ser. No. 08/699,678 (Atty. Docket No. EXT-002), filed on Aug. 14, 1997. Epithelial cells lining the colon normally migrate from a basal position in colonic crypts, where stem cells divide by mitosis, to the top of the crypts and are then shed into the lumen. Colonic epithelial cells that line the intestinal lumen typically undergo regeneration every four to five days as a result of the rapid turnover rate through the epithelium. Accordingly, sloughed epithelial cells or their DNA are constantly being deposited in the forming stool as it passes through the lumen. As the stool proceeds toward the rectum and becomes progressively more solid (from an initial liquid state), epithelial cells are only sloughed onto the portion of the stool making contact with the portion of the lumen that formerly contained those cells in its epithelial lining. Epithelial cells of a polyp undergo the same rapid life cycle and shedding described above for normal colonic epithelial cells. Accordingly, cells shed from polyps are typically only absorbed onto the surface of the forming stool that makes contact with the polyp. However, if the stool is in a liquid state, mixing of shed polyp cells throughout the stool occurs automatically.

Accordingly, the present invention provides methods for detecting genomic changes in a subpopulation of cells in a sample of biological material. Methods of the invention are useful for the detection of changes in the nucleotide sequence of an allele in a small subpopulation of cells present in a large, heterogeneous sample of diagnostically-irrelevant biological material.

Also, in a preferred embodiment, transformed cells sought to be detected using methods according to the invention are malignant cells. Transformed cells detected according to methods of the invention may be induced transformants, transformed, for example, by a virus, by radiation, or by chemical or other carcinogenic means.

Methods of the invention may be performed on any biological sample, including tissue and body fluid samples. Particularly preferred biological samples include pus, sputum, semen, blood, saliva, cerebrospinal fluid, and urine. In an important embodiment of the invention the sample is stool which is analyzed to detect colorectal cancer or precancer. Methods of the invention may be practiced by exposing a biological sample to one or more radionucleotides in order to separately detect the number X of a first polynucleotide and the number Y of a second polynucleotide. In a preferred embodiment the radionucleotides are incorporated into oligonucleotide probes which are exposed to the sample under conditions that promote specific hybridization of the radiolabeled oligonucleotide probes with the first or second polynucleotides. In a more preferred embodiment, unlabeled oligonucleotide probes are exposed to the sample. The probes are subsequently radiolabeled using a primer extension reaction in the presence of radio-

labeled nucleotides. The radiolabeled nucleotides are preferably chain terminating nucleotides, (e.g. dideoxynucleotides). The number of molecules of a polynucleotide in a sample is calculated from the measurement of the number of radioactive decay events that is specifically associated with the polynucleotide. The number of radioactive decay events is directly proportional to the number of molecules.

In a preferred embodiment the first and second radiolabeled oligonucleotides are separable from each other. For example, the first and second oligonucleotides are of different sizes and can be separated by gel electrophoresis, chromatography or mass spectrometry. In one embodiment the first and second oligonucleotides are of different lengths. In a preferred embodiment the size difference is imparted by a size marker which is specifically attached to one of the different size marks. Alternatively a different size marker is attached to each oligonucleotide. After separation, the number of radioactive decay events is measured for each oligonucleotide, and the number of molecules is calculated as described herein.

In a more preferred embodiment, the first and second oligonucleotides are of the same size but are labeled with different radioisotopes selected from, for example, ^{35}S, ^{32}P, ^{33}P, ^{3}H, ^{125}I and ^{14}C. The first and second oligonucleotides are then distinguished by different characteristic emission spectra. The number of radioactive decay events is measured for each oligonucleotide without separating the two oligonucleotides from each other.

Methods of the invention are especially useful for the detection of colorectal cancer or precancerous cells in humans. For purposes of the present invention, precancerous cells are cells that have a mutation that is associated with cancer, and which renders such cells susceptible to becoming cancerous. Such methods comprise determining whether cells or nucleotide debris in a stool sample include a deletion of a polynucleotide normally present in a wild-type genome of the human or other mammal. The sample may be exposed to a plurality of first and second oligonucleotide probes under hybridization conditions, thereby to hybridize (i) first probe to copies of a first polynucleotide segment characteristic of a wild-type genomic region known or suspected not to be deleted in cells of the sample and (ii) second probe to copies of a second polynucleotide segment characteristic of the wild-type genomic region suspected of being mutated in the sample. The number of duplexes formed with each of the first and second probes is then detected and counted. The presence of a statistically-significant difference in those two numbers is indicative of the presence in the sample of a mutation that may be characteristic of colorectal cancer. Endoscopy or other visual examination procedures are then indicated.

Methods according to the invention also may be used to detect a loss of heterozygosity at an allele by determination of the amounts of maternal and paternal alleles comprising a genetic locus that includes at least one single-base polymorphism. A statistically-significant difference in the numbers of each allele is indicative of a mutation in an allelic region encompassing the single-base polymorphism. In this method, a region of an allele comprising a single-base polymorphism is identified, using, for example, a database, such as GenBank, or by other means known in the art. Probes are designed to hybridize to corresponding regions on both paternal and maternal alleles immediately 3' to the single base polymorphism. After hybridization, a mixture of at least two of the four common dideoxy nucleotides are added to the sample, each labeled with a different detectable label. A DNA polymerase is also added. Using allelic DNA adjacent the polymorphic nucleotide as a template, hybridized probe is extended by the addition of a single dideoxynucleotide that is the binding partner for the polymorphic nucleotide. After washing to remove unincorporated dideoxynucleotides, the dideoxynucleotides which have been incorporated into the probe extension are detected by determining the number of bound extended probes bearing each of the two dideoxy nucleotides in, for example, a scintillation counter. The presence of an almost equal number of two different labels mean that there is normal heterozygosity at the polymorphic nucleotide. The presence of a statistically-significant difference between the detected numbers of the two labels means that a deletion of the region encompassing the polymorphic nucleotide has occurred in one of the alleles.

Methods of the invention may be used to determine whether a patient is a candidate for follow-up invasive diagnostic or other procedures, such as endoscopy. For example, methods of the invention may be used to detect a mutation in a tumor suppressor gene or an oncogene in a subpopulation of cells in a stool sample obtained from a patient. An endoscopy procedure may then be performed on patients diagnosed with a mutation. A positive endoscopy result is then followed by polypectomy, surgery, or other treatment to remove cancerous or precancerous tissue.

Accordingly, it is an object of the invention to provide methods for detecting loss of heterozygosity in a subpopulation of cells in a cellular sample. It is a further object of the invention to provide methods for detecting a genomic change in a subpopulation of cells, wherein the genomic change is indicative of cancer. It is another object of the invention to detect a loss of heterozygosity in a genomic region associated with cancer, such as a tumor suppressor region. It is yet another object of the invention to provide methods for detecting heterozygosity and the loss thereof at single-base polymorphic nucleic acids. Finally, it is an object of the invention to provide methods for the detection of cancer, and particularly colorectal cancer by detection of cells or cellular debris indicative of cancer in a heterogeneous sample, such as a stool sample.

Further aspects of the invention will become apparent upon consideration of the following detailed description and of the drawings.

DESCRIPTION OF THE DRAWINGS

FIG. **1** depicts differential primer extension as exemplified below.

FIGS. **2**A and **2**B are model Gaussian distributions showing regions of low statistical probability.

FIG. **3** is graph showing the probable values of N for a heterogeneous population of cells in which 1% of the cells are mutated.

DETAILED DESCRIPTION OF THE INVENTION

Methods according to the present invention are useful for the detection of loss of heterozygosity in a heterogeneous cellular sample in which the loss of heterozygosity occurs in only a small subpopulation of cells in the sample. Using traditional detection methods, such a subpopulation would be difficult, if not impossible, to detect especially if the deletion end points are unknown at the time of detection or a clonally-impure cellular population is used. See, e.g., U.S. Pat. No. 5,527,676 (reporting that a clonal population of

cells should be used in order to detect a deletion in a p53 gene). Traditional methods for detection of mutations involved in carcinogenesis rely upon the use of a clonally-pure population of cells and such methods are best at detecting mutations that occur at known "hot spots" in oncogenes, such as k-ras. See, Sidransky, supra.

Methods of the present invention are useful for detecting loss of heterozygosity in a small number of cells in an impure cellular population because such methods do not rely upon knowing the precise deletion end-points and such methods are not affected by the presence in the sample of heterogeneous DNA. For example, in loss of heterozygosity, deletions occur over large portions of the genome and entire chromosome arms may be missing. Methods of the invention comprise counting a number of molecules of a target nucleic acid suspected of being deleted and comparing it to a reference number. In a preferred embodiment the reference number is the number of molecules of a nucleic acid suspected of not being deleted in the same sample. All that one needs to know is at least a portion of the sequence of a target nucleic acid suspected of being deleted and at least a portion of the sequence of a reference nucleic acid suspected of not being deleted. Methods of the invention, while amenable to multiple mutation detection, do not require multiple mutation detection in order to detect indicia of cancer in a heterogeneous sample.

Accordingly, methods of the present invention are useful for the detection of loss of heterozygosity in a subpopulation of cells or debris therefrom in a sample. Loss of heterozygosity generally occurs as a deletion of at least one wild-type allelic sequence in a subpopulation of cells. In the case of a tumor suppressor gene, the deletion typically takes the form of a massive deletion characteristic of loss of heterozygosity. Often, as in the case of certain forms of cancer, disease-causing deletions initially occur in a single cell which then produces a small subpopulation of mutant cells. By the time clinical manifestations of the mutation are detected, the disease may have progressed to an incurable stage. Methods of the invention allow detection of a deletion when it exists as only a small percentage of the total cells or cellular debris in a sample.

Methods of the invention comprise a comparison of the number of molecules of two nucleic acids that are expected to be present in the sample in equal numbers in normal (non-mutated) cells. In a preferred embodiment, the comparison is between (1) an amount of a genomic polynucleotide segment that is known or suspected not to be mutated in cells of the sample (the "reference") and (2) an amount of a wild-type (non-mutated) genomic polynucleotide segment suspected of being mutated in a subpopulation of cells in the sample (the "target"). A statistically-significant difference between the amounts of the two genomic polynucleotide segments indicates that a mutation has occurred.

In a preferred embodiment, the reference and target nucleic acids are alleles of the same genetic locus. Alleles are useful in methods of the invention if there is a sequence difference which distinguishes one allele from the other. In a preferred embodiment, the genetic locus is on or near a tumor suppressor gene. Loss of heterozygosity can result in loss of either allele, therefore either allele can serve as the reference allele. The important information is the presence or absence of a statistically significant difference between the number of molecules of each allele in the sample. Also in a preferred embodiment, the reference and target nucleic acids are different genetic loci, for example different genes. In a preferred embodiment, the reference nucleic acid comprises both alleles of a reference genetic locus and the target nucleic acid comprises both alleles of a target genetic locus, for example a tumor suppressor gene. Specifically, in the case of a deletion in a tumor suppressor gene, the detected amount of the reference gene is significantly greater than the detected amount of the target gene. If a target sequence is amplified, as in the case of certain oncogene mutations, the detected amount of target is greater than the detected amount of the reference gene by a statistically-significant margin.

Methods according to the art generally require the use of numerous probes, usually in the form of PCR primers and/or hybridization probes, in order to detect a deletion or a point mutation. However, because methods of the present invention involve enumerative detection of nucleotide sequences and enumerative comparisons between sequences that are known to be stable and those that are suspected of being unstable, only a few probes must be used in order to accurately assess cancer risk. In fact, a single set (pair) of probes is all that is necessary to detect a single large deletion. The risk of cancer is indicated by the presence of a mutation in a genetic region known or suspected to be involved in oncogenesis. Patients who are identified as being at risk based upon tests conducted according to methods of the invention are then directed to other, typically invasive, procedures for confirmation and/or treatment of the disease.

Enumerative sampling of a nucleotide sequence that is uniformly distributed in a biological sample typically follows a Poisson distribution. For large populations, such as the typical number of genomic polynucleotide segments in a biological sample, the Poisson distribution is similar to a normal (Gaussian) curve with a mean, N, and a standard deviation that may be approximated as the square root of N.

Statistically-significance between numbers of target and reference genes obtained from a biological sample may be determined by any appropriate method. See, e.g., Steel, et al., Principles and Procedures of Statistics, A Biometrical Approach (McGraw-Hill, 1980), the disclosure of which is incorporated by reference herein. An exemplary method is to determine, based upon a desired level of specificity (tolerance of false positives) and sensitivity (tolerance of false negatives) and within a selected level of confidence, the difference between numbers of target and reference genes that must be obtained in order to reach a chosen level of statistical significance. A threshold issue in such a determination is the minimum number, N, of genes (for each of target and reference) that must be available in a population in order to allow a determination of statistical significance. The number N will depend upon the assumption of a minimum number of mutant alleles in a sample containing mutant alleles (assumed herein to be at least 1%) and the further assumption that normal samples contain no mutant alleles. It is also assumed that a threshold differences between the numbers of reference and target genes must be at least 0.5% for a diagnosis that there is a mutation present in a subpopulation of cells in the sample. Based upon the foregoing assumptions, it is possible to determine how large N must be so that a detected difference between numbers of mutant and reference alleles of less than 0.5% is truly a negative (i.e. no mutant subpopulation in the sample) result 99.9% of the time.

The calculation of N for specificity, then, is based upon the probability of one sample measurement being in the portion of the Gaussian distribution covering the lowest 3.16% of the population (the area marked "A" in FIG. **2**A) and the probability that the other sample measurement is in the portion of the Gaussian distribution covering the highest 3.16% of the population (the area marked "B" in FIG. **2**B). Since the two sample measurements are independent events,

the probability of both events occurring simultaneously in a single sample is approximately 0.001 or 0.1%. Thus, 93.68% of the Gaussian distribution (100%−2×3.16%) lies between the areas marked A and B in FIG. **3**. Statistical tables indicate that such area is equivalent to 3.72 standard deviations. Accordingly, 0.5% N is set equal to 3.72 sigma. Since sigma (the standard deviation) is equal to $\sqrt{N}$, the equation may be solved for N as 553,536. This means that if the lower of the two numbers representing reference and target is at least 553,536 and if the patient is truly normal, the difference between the numbers will be less than 0.5% about 99.9% of the time.

To determine the minimum N required for 99% sensitivity a similar analysis is performed. This time, one-tailed Gaussian distribution tables show that 1.28 standard deviations (sigma) from the mean cover 90% of the Gaussian distribution. Moreover, there is a 10% (the square root of 1%) probability of one of the numbers (reference or target) being in either the area marked "A" in FIG. **3** or in the area marked "B" in FIG. **3**. If the two population means are a total of 1% different and if there must be a 0.5% difference between the number of target and reference genes, then the distance from either mean to the threshold for statistical significance is equivalent to 0.25% N (See FIG. **3**) for 99% sensitivity. As shown in FIG. **3**, 0.25% N corresponds to about 40% of one side of the Gaussian distribution. Statistical tables reveal that 40% of the Gaussian distribution corresponds to 1.28 standard deviations from the mean. Therefore, 1.28 sigma is equal to 0.0025N, and N equals 262,144. Thus, for abnormal samples, the difference will exceed 0.5% at least 99% of the time if the lower of the two numbers is at least 262,144. Conversely, an erroneous negative diagnosis will be made only 1% of the time under these conditions.

In order to have both 99.9% specificity (avoidance of false positives) and 99% sensitivity (avoidance of false negatives), a sample with DNA derived from at least 553, 536 (or roughly greater than 550,000) cells should be counted. A difference of at least 0.5% between the numbers obtained is significant at a confidence level of 99.0% for sensitivity and a difference of less than 0.5% between the numbers is significant at a confidence level of 99.9% for specificity. As noted above, other standard statistical tests may be used in order to determine statistical significance and the foregoing represents one such test.

Based upon the foregoing explanation, the skilled artisan appreciates that methods of the invention are useful to detect mutations in a subpopulation of a polynucleotides in any biological sample. For example, methods disclosed herein may be used to detect allelic loss (the loss of heterozygosity) associated with diseases such as cancer. Additionally, methods of the invention may be used to detect a deletion or a base substitution mutation causative of a metabolic error, such as complete or partial loss of enzyme activity. For purposes of exemplification, the following provides details of the use of methods according to the present invention in colon cancer detection. Inventive methods are especially useful in the early detection of a mutation (and especially a large deletion typical of loss of heterozygosity) in a tumor suppressor gene. Accordingly, while exemplified in the following manner, the invention is not so limited and the skilled artisan will appreciate its wide range of applicability upon consideration thereof.

Methods according to the invention preferably comprise comparing a number of a target polynucleotide known or suspected to be mutated to a number of a reference polynucleotide known or suspected not to be mutated. In addition to the alternative embodiments using either alleles or genetic loci as reference and target nucleic acids, the invention comprises a comparison of a microsatellite repeat region in a normal allele with the corresponding microsatellite region in an allele known or suspected to be mutated. Exemplary detection means of the invention comprise determining whether a difference exists between the number of counts of each nucleic acid being measured. The presence of a statistically-significant difference is indicative that a mutation has occurred in one of the nucleic acids being measured.

I. Preparation of a Stool Sample

A sample prepared from stool voided by a patient should comprise at least a cross-section of the voided stool. As noted above, stool is not homogenous with respect to sloughed cells. As stool passes through the colon, it absorbs sloughed cells from regions of the colonic epithelium with which it makes contacts. Thus, sloughed cells from a polyp are absorbed on only one surface of the forming stool (except near the cecum where stool is still liquid and is homogenized by Intestinal Peristalsis). Taking a representative sample of stool (i.e., at least a cross-section) and homogenizing it ensures that sloughed cells from all epithelial surfaces of the colon will be present for analysis in the processed stool sample. Stool is voided into a receptacle that is preferably small enough to be transported to a testing facility. The receptacle may be fitted to a conventional toilet such that the receptacle accepts stool voided in a conventional manner. The receptacle may comprise a mesh or a screen of sufficient size and placement such that stool is retained while urine is allowed to pass through the mesh or screen and into the toilet. The receptacle may additionally comprise means for homogenizing voided stool. Moreover, the receptacle may comprise means for introducing homogenization buffer or one or more preservatives, such as alcohol or a high salt concentration solution, in order to neutralize bacteria present in the stool sample and to inhibit degradation of DNA.

The receptacle, whether adapted to fit a toilet or simply adapted for receiving the voided stool sample, preferably has sealing means sufficient to contain the voided stool sample and any solution added thereto and to prevent the emanation of odors. The receptacle may have a support frame which is placed directly over a toilet bowl. The support frame has attached thereto an articulating cover which may be placed in a raised position, for depositing of sample or a closed position (not shown) for sealing voided stool within the receptacle. The support frame additionally has a central opening traversing from a top surface through to a bottom surface of the support frame. The bottom surface directly communicates with a top surface of the toilet. Extending from the bottom surface of the support frame and encompassing the entire circumference of the central opening is a means for capturing voided stool. The means for capturing voided stool may be fixedly attached to the support frame or may be removably attached for removal subsequent to deposition of stool.

Once obtained, the stool sample is homogenized in an appropriate buffer, such as phosphate buffered saline or a chaotropic salt solution. Homogenization means and materials for homogenization are generally known in the art. See, e.g., U.S. Pat. No. 4,101,279. Thus, particular homogenization methods may be selected by the skilled artisan. Methods for further processing and analysis of a biological sample, such as a stool sample are presented below.

II. Methods for Detection of Colon Cancer or Precancer

For exemplification, methods of the invention are used to detect a deletion or other mutation in or near the p53 tumor suppressor gene in cells obtained from a representative stool

sample. The p53 gene is a good choice because the loss of heterozygosity in p53 is often associated with colorectal cancer. An mRNA sequence corresponding to the DNA coding region for p53 is reported as GenBank Accession No. M92424. The skilled artisan understands that methods described herein may be used to detect mutations in any gene and that detection of a p53 deletion is exemplary of such methods. In the detection of loss of heterozygosity, it is not necessary to target any particular gene due to the massive deletions associated with this event. Accordingly, an LOH-type deletion involving, for example, p53 may be detected by probing a region outside, but near, p53 because that region is also likely to be deleted. At least a cross-section of a voided stool sample is obtained and prepared as described immediately above. DNA or RNA may optionally be isolated from the sample according to methods known in the art. See, Smith-Ravin, et al., *Gut,* 36: 81–86 (1995), incorporated by reference herein. Methods of the invention may also comprise the step of amplifying DNA or RNA sequences using the polymerase chain reaction. However, methods of the invention may be performed on unprocessed stool.

Nucleic acids may be sheared or cut into small fragments by, for example, restriction digestion. The size of nucleic acid fragments produced is not critical, subject to the limitations described below. A target nucleic acid that is suspected of being mutated (p53 in this example) and a reference nucleic acid are chosen. The target and reference nucleic acids may be alleles on or near the p53 gene. Alternatively, the target nucleic acid comprises both alleles on or near the p53 gene and the reference nucleic acid comprises both alleles on or near a genetic locus suspected not to be deleted. Single-stranded nucleic acid fragments may be prepared using well-known methods. See, e.g., Sambrook, et al., *Molecular Cloning, A Laboratory Manual* (1989) incorporated by reference herein.

Either portions of a coding strand or its complement may be detected in methods according to the invention. In a preferred embodiment, both first and second strands of an allele are present in a sample during hybridization to an oligonucleotide probe. The sample is exposed to an excess of probe that is complementary to a portion of the first strand, under conditions to promote specific hybridization of the probe to the portion of the first strand. In a most preferred embodiment, the probe is in sufficient excess to bind all the portion of the first strand, and to prevent reannealing of the first strand to the second strand of the allele. Also in a preferred embodiment, the second strand of an allele is removed from a sample prior to hybridization to an oligonucleotide probe that is complementary to a portion of the first strand of the allele. For exemplification, detection of the coding strand of p53 and reference allele are described. Complement to both p53 and reference allele are removed by hybridization to anti-complement oligonucleotide probes (isolation probes) and subsequent removal of duplex formed thereby. Methods for removal of complement strands from a mixture of single-stranded oligonucleotides are known in the art and include techniques such as affinity chromatography. Upon converting double-stranded DNA to single-stranded DNA, sample is passed through an affinity column comprising bound isolation probe that is complementary to the sequence to be isolated away from the sample. Conventional column chromatography is appropriate for isolation of complement. An affinity column packed with sepharose or any other appropriate materials with attached complementary nucleotides may be used to isolate complement DNA in the column, while allowing DNA to be analyzed to pass through the column. See Sambrook, Supra. As an alternative, isolation beads may be used to exclude complement as discussed in detail below.

After removal of complement, the target and reference nucleic acids are exposed to radio-labeled nucleotides under conditions which promote specific association of the radio-labeled nucleotides with the target and reference nucleic acids in a sample. In order to count the number of molecules of the target and reference nucleic acids, the radionucleotides associated with the target nucleic acid must be distinguished from the radionucleotides associated with the reference nucleic acid. In addition, the radionucleotides that are specifically associated with either target or reference nucleic acid must be distinguished from radionucleotides that are not associated with either nucleic acid. The number of molecules of target nucleic acid is counted by measuring a number X of radioactive decay events (e.g. by measuring the total number of counts during a defined interval or by measuring the time it takes to obtain a predetermined number of counts) specifically associated with the target nucleic acid. The number X is used to calculate the number X1 of radionucleotides which are specifically associated with the target nucleic acid. The number X1is used to calculate the number X2 of target nucleic acid molecules, knowing the ratio of radionucleotide molecules to target nucleic acid molecules in the assay.

According to methods of the invention, it is important to count the number of molecules in order to provide a statistical analysis of the likelihood of loss of heterozygosity. Comparison of the numbers of radioactive decays without knowing the numbers of molecules associated with the radioactive decays does not provide statistical data on the significance of any observed difference.

In a preferred embodiment, a radionucleotide is incorporated into a specific oligonucleotide prior to exposure to the sample. In a most preferred embodiment, a radiolabeled oligonucleotide is used which comprises a single radionucleotide molecule per oligonucleotide molecule. A radiolabeled oligonucleotide is designed to hybridize specifically to a target nucleic acid. In one embodiment the target nucleic acid is a specific allele of a polymorphic genetic locus, and the oligonucleotide is designed to be complementary to the allele at the site of polymorphism. One skilled in the art can perform hybridizations under conditions which promote specific hybridization of the oligonucleotide to the allele, without cross hybridizing to other alleles. Similarly, radiolabeled oligonucleotides are designed to specifically hybridize with the reference nucleic acid.

Also in a preferred embodiment, a radionucleotide is specifically incorporated into an oligonucleotide by primer extension, after exposing the oligonucleotide to the sample under conditions to promote specific hybridization of the oligonucleotide with the target nucleic acid. In a preferred embodiment the oligonucleotide is unlabeled, and the radionucleotide is a radiolabeled chain terminating nucleotide (e.g. a dideoxynucleotide). In a most preferred embodiment, the radionucleotide is the chain terminating nucleotide complementary to the nucleotide immediately 5' to the nucleotide that base pairs to the 3' nucleotide of the oligonucleotide when it is specifically hybridized to the target nucleic acid. In the embodiment where the target nucleic acid is an allele of a polymorphic genetic locus, the oligonucleotide is preferably designed such that the 3' nucleotide of the oligonucleotide base pairs with the nucleotide immediately 3' to the polymorphic residue. In a preferred embodiment, a radiolabeled terminating nucleotide that is complementary to the residue at the polymorphic site is

incorporated on the 3' end of the specifically hybridized oligonucleotide by a primer extension reaction. Similarly, in a preferred embodiment, a radionucleotide is specifically associated with a reference nucleic acid by primer extension. Other methods for specifically associating a radioactive isotope with a target or reference nucleic acid (for example a radiolabeled sequence specific DNA binding protein) are also useful for the methods of the invention.

In a preferred embodiment, prior to counting the radioactive decay events, the radionucleotides specifically associated with target and reference nucleic acids are separated from the radionucleotides that are not specifically associated with either nucleic acid. Separation is performed as described herein, or using techniques known in the art. Other separation techniques are also useful for practice of the invention. Methods of the invention also comprise distinguishing the radio-label specifically associated with a target nucleic acid from the radio-label specifically associated with a reference nucleic acid. In a preferred embodiment the isotope associated with the target is different from the isotope associated with the receptor. Different isotopes useful to radio-label nucleotides include ^{35}S, ^{32}P, ^{33}P, ^{125}I, ^{3}H, and ^{14}C. In one embodiment, an oligonucleotide complementary to a target nucleic acid is labeled with a different isotope from an oligonucleotide complementary to a reference nucleic acid. In another embodiment, the chain terminating nucleotide associated with the target nucleic acid is different from the chain terminating nucleotide associated with the reference nucleic acid, and the two chain terminating nucleotides are labeled with different isotopes.

In a preferred embodiment, radionucleotides labeled with different isotopes are detected without separating the radionucleotide associated with the target nucleic acid from the radionucleotide associated with the reference nucleic acid. The different isotopes useful to the invention have different characteristic emission spectra. The presence of a first isotope does not prevent the measurement of radioactive decay events of a second isotope. In a more preferred embodiment, the labeled oligonucleotide associated with the target nucleic acid is the same size as the labeled oligonucleotide associated with the reference nucleic acid (the labeled oligonucleotides can be labeled prior to hybridization or by primer extension). The two differentially labeled oligonucleotides are electrophoresed on a gel, preferably a denaturing gel, and the gel is exposed to an imager that detects the radioactive decay events of both isotopes. In this embodiment the two isotopes are detected at the same position on the imager, because both oligonucleotides migrate to the same position on the gel. Detection at the same position on the imager reduces variation due to different detection efficiencies at different positions on the imager.

Also in a preferred embodiment, the radionucleotide associated with the target nucleic acid is separated from the radionucleotide associated with the reference nucleic acid prior to measuring radioactive decay events. In a preferred embodiment the separated radionucleotides are labeled with the same isotope.

Preferred separation methods comprise conferring different molecular weights to the radionucleotides specifically associated with the target and reference nucleic acids.

In a preferred embodiment, first probes comprise a "separation moiety." Such separation moiety is, for example, hapten, biotin, or digoxigenin. The separation moiety in first probes does not interfere with the first probe's ability to hybridize with template or be extended. In an alternative embodiment, the labeled ddNTPs comprise a separation moiety. In yet another alternative embodiment, both the first probes and the labeled ddNTPs comprise a separation moiety. Following the extension reaction, a high molecular weight molecule having affinity for the separation moiety (e.g., avidin, streptavidin, or anti-digoxigenin) is added to the reaction mixture under conditions which permit the high molecular weight molecule to bind to the separation moiety. The reaction components are then separated on the basis of molecular weight using techniques known in the art such as gel electrophoresis, chromatography, or mass spectroscopy. See, Ausubel et al., *Short Protocols in Molecular Biology,* 3rd ed. (John Wiley & Sons, Inc., 1995); *Wu Recombinant DNA Methodology II,* (Academic Press, 1995).

Also in a preferred embodiment the radionucleotide associated with a first allele of a polymorphic genetic locus is separated from the radionucleotide associated with a second allele of the polymorphic locus by differential primer extension, wherein the extension products of a given oligonucleotide primer are of a different length for each of the two alleles. In differential primer extension (exemplified in FIG. **1**) an oligonucleotide is hybridized such that the 3' nucleotide of the oligonucleotide base pairs with the nucleotide that is immediately 5' of the polymorphic site. The extension reaction is performed in the presence of a radiolabeled terminator nucleotide complementary to the nucleotide at the polymorphic site of the first allele. The reaction also comprises non-labeled nucleotides complementary to the other 3 nucleotides. Extension of a primer hybridized to the first allele results in a product having only the terminator nucleotide incorporated (exemplified in FIG. **1A**, T* is the labeled terminator nucleotide). Extension of a primer hybridized to the second allele results in a product that incorporates several non-labeled nucleotides immediately 5' to the terminator nucleotide (exemplified in FIG. **1B**). The number of non-labeled nucleotides that are incorporated is determined by the position, on the template nucleic acid, of the closest 5' nucleotide complementary to the terminator nucleotide. In an alternative embodiment, differential primer extension comprises a labeled oligonucleotide and a non-labeled terminator nucleotide.

Labeled probes are exposed to sample under hybridization conditions. Such conditions are well-known in the art. See, e.g., Wallace, et al., *Nucleic Acids Res.,* 6: 3543–3557 (1979), incorporated by reference herein. First and Second oligonucleotide probes that are distinctly labeled (i.e. with different radioactive isotopes, fluorescent means, or with beads of different size) are applied to a single aliquot of sample. After exposure of the probes to sample under hybridization conditions, sample is washed to remove any unhybridized probe. Thereafter, hybridized probes are detected separately for p53 hybrids and reference allele hybrids. Standards may be used to establish background and to equilibrate results. Also, if differential fluorescent labels are used, the number of probes may be determined by counting differential fluorescent events in a sample that has been diluted sufficiently to enable detection of single fluorescent events in the sample. Duplicate samples may be analyzed in order to confirm the accuracy of results obtained.

If there is a difference between the amount of p53 detected and the amount of the reference allele detected greater than a 0.5% difference with at least 550,000 events (earlier shown to be the threshold of significance), it may be assumed that a mutation has occurred in the region involving p53 and the patient is at risk for developing or has developed colon cancer. Statistical significance may be determined by any known method. A preferred method is outlined above.

The determination of a p53 mutation allows a clinician to recommend further treatment, such as endoscopy procedures, in order to further diagnose and, if necessary, treat the patient's condition. The following examples illustrate methods of the invention that allow direct quantification of hybridization events.

What is claimed is:

1. A method for detecting the presence of a mutant nucleic acid in a sample population, comprising the steps of:

a) introducing a first radionucleotide to a sample population suspected to contain a subpopulation of a nucleic acid mutant, wherein said first radionucleotide hybridizes to a first wild-type nucleic acid target, a subpopulation of which suspected to be mutated in the sample;

b) introducing a second radionucleotide to the sample, wherein said second radionucleotide hybridizes to a second wild-type nucleic acid target in the sample

c) washing said sample to remove unhybridized first and second radionucleotides;

d) determining a number X of radioactive decay events associated with said first radionucleotide;

e) determining a number Y of radioactive decay events associated with said second radionucleotide;

f) determining whether a difference exists between number X and number Y, the presence of a statistically-significant difference being indicative of the presence of a mutation in said sample.

2. The method of claim **1** wherein said first radionucleotide is capable of hybridizing to a nucleic acid in the sample that is suspected to be mutated in cancer or precancer; and said second radionucleotide is capable of hybridizing to a nucleic acid in the sample that is not mutated in cancer or precancer.

3. The method of claim **1** wherein said first radionucleotide is capable of hybridizing to a portion of the maternal allele at a genetic locus; and said second radionucleotide is capable of hybridizing to a portion of the paternal allele at said locus.

4. The method of claim **1** further comprising the step of isolating said first radionucleotide specifically bound to a first target nucleic acid, and said second radionucleotide specifically bound to a second target nucleic acid.

5. The method of claim **4** wherein said isolating step is selected from the group consisting of gel electrophoresis, chromatography, and mass spectrometry.

6. The method of claim **4** wherein said number X is correlated with a number X1 of molecules of said first nucleic acid, and said number Y is correlated with a number Y1 of molecules of said second nucleic acid.

7. The method of claim **1** wherein at least one of said first and second radionucleotides is a chain terminator nucleotide.

8. The method of claim **1** wherein at least one of said first and second radionucleotides is an oligonucleotide.

9. The method of claim **1** wherein said radionucleotides are labeled with an isotope selected from the group consisting of 32P, 33P, 35S, 125I and 14C.

10. The method of claim **1** wherein each of said first and second radionucleotides are labeled with a different isotope.

11. The method of claim **10** wherein said numbers X and Y are determined by coincidence counting.

12. A method for determining the number of molecules of a nucleic acid comprising the steps of:

a) exposing a sample to a plurality of first radionucleotides;

b) isolating radionucleotides specifically bound to first target nucleic acid molecules;

c) determining a number of radioactive decay events associated with the radionucleotides of step b);

d) calculating a number of molecules of said sequence as equivalent to said number of radioactive decay events.

13. A method for detecting the presence of a mutation in a nucleic acid, comprising the steps of:

a) exposing a sample to a plurality of a oligonucleotide;

b) performing a primer extension reaction in the presence of a plurality of a chain terminating nucleotide, to generate extension products of said oligonucleotide;

c) determining the size of the extension products, the presence of extension products of different sizes being indicative of the presence of a mutation.

14. The method of claim **13** wherein said oligonucleotide is capable of hybridizing to a member selected from the group consisting of a maternal allele and a paternal allele of the same genetic locus.

15. The method of claim **13** wherein said oligonucleotide is labeled.

16. The method of claim **13** wherein said terminating nucleotide is labeled.

17. The method of claims **15** or **16** wherein said label is a radioactive isotope.

18. The method of claim **13** wherein said extension reaction is performed in the presence of at least two differentially labeled chain terminating nucleotides.

19. A method for detecting loss of heterozygosity in a nucleic acid, comprising the steps of:

a) contacting a sample with a radionucleotide;

b) isolating a nucleic acid specifically bound to said radionucleotide;

c) determining a number of radioactive decay events associated with said nucleic acid;

d) comparing said number to a reference number,

wherein a statistically significant difference between said number and said reference number is indicative of loss of heterozygosity.

20. The method of claim **1**, wherein said sample comprises cellular material from a population of patients.

21. The method of claim **20**, wherein said population of patients is healthy.

22. The method of claim **20**, wherein said population of patients has a disease suspected to be associated with said mutant nucleic acid.

23. The method of claim **20**, wherein said disease is cancer.

24. The method of claim **1**, wherein said mutant nucleic acid is an allelic variant.

25. The method of claim **24**, wherein said variant is a single nucleotide polymorphism.

* * * * *

UNITED STATES PATENT AND TRADEMARK OFFICE

CERTIFICATE OF CORRECTION

PATENT NO. : 5,928,870
APPLICATION NO. : 08/876857
DATED : July 27, 1999
INVENTOR(S) : Stanley Lapidus et al.

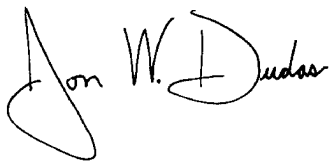
It is certified that error appears in the above-identified patent and that said Letters Patent is hereby corrected as shown below:

Column 1, between line 2, ending "HETEROZYGOSITY", and line 4, beginning "Field of", insert the following paragraph:

--This application is a continuation-in-part of U.S. Ser. No. 08/700,583, filed on August 14, 1996, now U.S. Pat. No. 5,670,325.--

Signed and Sealed this

Thirtieth Day of December, 2008



JON W. DUDAS
Director of the United States Patent and Trademark Office

EXHIBIT 7

US007915015B2

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

(10) **Patent No.: US 7,915,015 B2**
(45) **Date of Patent: *Mar. 29, 2011**

(54) **DIGITAL AMPLIFICATION**

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

(73) Assignee: **The 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.

This patent is subject to a terminal disclaimer.

(21) Appl. No.: **12/617,368**

(22) Filed: **Nov. 12, 2009**

(65) **Prior Publication Data**

US 2010/0209921 A1 Aug. 19, 2010

Related U.S. Application Data

(60) Division of application No. 11/709,742, filed on Feb. 23, 2007, now Pat. No. 7,824,889, which is a continuation of application No. 10/828,295, filed on Apr. 21, 2004, now abandoned, which is a division of application No. 09/981,356, filed on Oct. 12, 2001, now Pat. No. 6,753,147, which is a continuation of application No. 09/613,826, filed on Jul. 11, 2000, now Pat. No. 6,440,706.

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

(51) **Int. Cl.**
C12P 19/34 (2006.01)
C07H 21/04 (2006.01)

(52) **U.S. Cl.** **435/91.2**; 536/24.3; 536/24.31; 536/24.33

(58) **Field of Classification Search** ..... None
See application file for complete search history.

(56) **References Cited**

U.S. PATENT DOCUMENTS

5,213,961	A	5/1993	Bunn et al.
5,518,901	A	5/1996	Murtagh
5,670,325	A	9/1997	Lapidus et al.
5,736,333	A	4/1998	Livak 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.
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.
6,143,496	A	11/2000	Brown et al.

(Continued)

FOREIGN PATENT DOCUMENTS

EP 0643140 3/1995

(Continued)

OTHER PUBLICATIONS

Loughlin et al., "Association of the Interleukin-1 Gene Cluster on Chromosome 2q13 With Knee Osteoarthritis," Arthritis & Rheumatism, Jun. 2002, 46(6):1519-1527.

(Continued)

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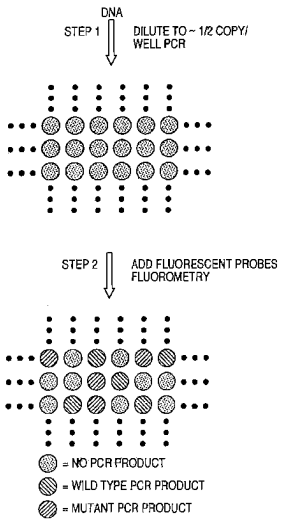
(57) **ABSTRACT**

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 pre-defined mutations. The process provides a reliable and quantitative measure of the proportion of variant sequences within a DNA sample.

18 Claims, 7 Drawing Sheets



U.S. PATENT DOCUMENTS

6,291,163 B1 9/2001 Sidransky

FOREIGN PATENT DOCUMENTS

WO	95/13399	5/1995
WO	99/13113	3/1999

OTHER PUBLICATIONS

P. J. Sykes, "Quantitation of Targets for PCR by Use of Limiting Dilution," BioTechniques, 1992, vol. 13, No. 3, pp. 444-449.

A. Piatek et al., "Molecular Beacon Sequence Analysis for Detecting Drug Resistance in Mycobacterium Tuberculosis," Nature Biotechnology, Apr. 1998, vol. 16, No. 4. pp. 359-363.

S. Tyagi et al., "Multicolor Molecular Beacons for allele discrimination," Nature Biotechnology, Jan. 1998, vol. 16, No. 1, pp. 303-308.

J. A.M. Vet et al., "Multilex Detection of Four Pathogenic Retroviruses Using Molecular Beacons," Proceedings of the National Academy of Sciences of the United States, May 25, 1999, vol. 96, No. 11, pp. 6394-6399.

S. Tyagi et al., "Molecular Beacons: probes that Fluoresce Upon Hybridization," Nature Biotechnology, 1996, vol. 14, No. 3, pp. 303-308.

W. P. Halford et al., "The Inherent Quantitative Capacity of the Reverse Transcription-Polymerase Chain Reaction," Analytical Biochemistry, Jan. 15, 1999, vol. 266, No. 2, pp. 181-191.

B. Vogelstein et al., "Digital PCR," Proceedings of the National Academy of Sciences of the United States, Aug. 3, 1999, vol. 96, No. 16, pp. 9236-9241.

K. D.E. Everett et al., "Identification of Nine Species of the Chlamydiaceae Using PCR-RFLP," Int. J. Syst. Bacteriol., Apr. 1999, vol. 49, No. 2, pp. 803-813.

D. G. Monckton et al., "Minisatellite "Isoallele" Discrimination in Pseudohomozygotes by Single Molecule PCR and Variant Repeat Mapping," Genomics, 1991, vol. 11, pp. 465-467.

G. Ruano et al., "Haplotype of Multiple Polymorphisms Resolved by Enzymatic Amplification of Single DNA Molecules," Proc. National Science USA, 1990, pp. 6296-6300.

W. Navidi et al., "Using PCR in Preimplantation Genetic Disease Diagnosis," Human Reproduction, 1991, vol. 6, No. 6, pp. 836-849.

H. Li et al., "Amplification and Analysis of DNA Sequences in Single Human Sperm and Diploid Cells," Nature, Sep. 29, 1988, vol. 335, pp. 414-417.

L. Zhang et al., "Whole Genome Amplification from a Single Cell: Implications for Genetic Analysism," Proc. National Science USA, Jul. 1992, vol. 89, pp. 5847-5851.

D. Sidransky et al., "Clonal Expansion of p53 Mutant Cells is Associated with Brain Tumour Progression," Nature, Feb. 27, 1992, pp. 846-847.

A. J. Jeffreys et al., "Mutation Processes at Human Minisatellites," Electophoresis, 1995, pp. 1577-1585.

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.

P. M. Lizardi et al., "Mutation Detection and Single-Molecule Counting Using Isothermal Rolling-Circle Amplification," Nature Genetics, Jul. 1998, vol. 19, pp. 225-232.

R. Parsons et al., "Mismatch Repair Deficiency in Phenotypically Normal Human Cells," Science, May 5, 1995, vol. 268, pp. 738-740.

Marras et al., "Multiplex Detection of Single-Nucleotide Variations Using Molecular Beacons," Genetic Analysis: Biomolecular Engineering, Feb. 1999, Vo. 14, pp. 151-156.

Whitcomb et al., "Detection of PCR Products Using Self-Probing Amplicons and Fluorescence," Nature Biotechnology, Aug. 1999, vol. 17, pp. 804-807.

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.

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, Jan. 22, 1994, vol. 343, pp. 196-200.

Notice of Reasons for Rejection dispatched Apr. 28, 2010 in Japanese Application No. 2001-513641 and English translation thereof.

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).

Newton, PCR Essential Data, pp. 51-52, 1995.

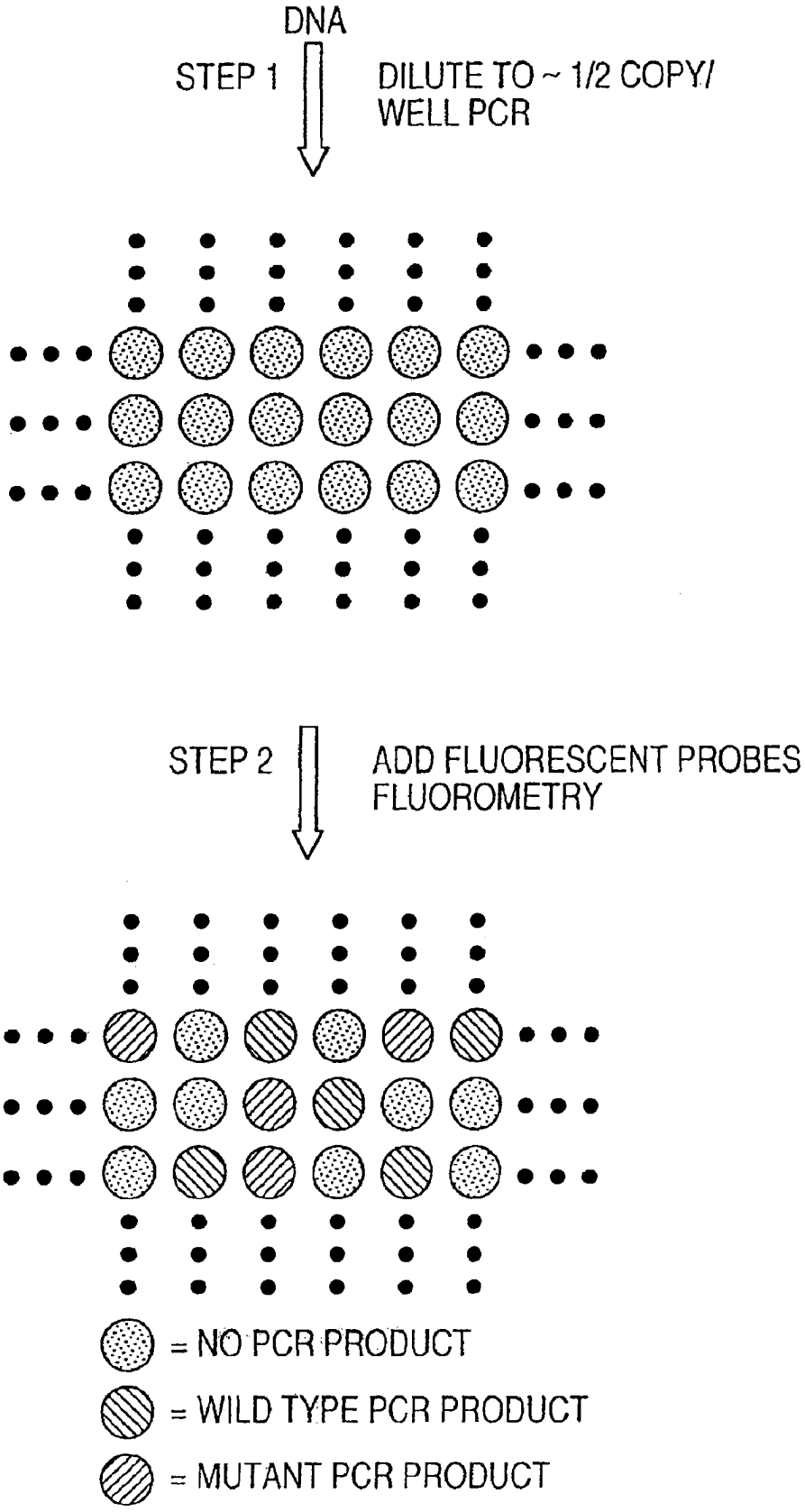
Office Action dated Jun. 11, 2010, in co-pending U.S. Appl. No. 11/709,742.

Office Action dated Dec. 29, 2009 in co-pending U.S. Appl. No. 11/709,742.

Office Action dated Sep. 18, 2009 in co-pending U.S. Appl. No. 11/709,742.

Office Action dated Jun. 5, 2009 in co-pending U.S. Appl. No. 11/709,742.

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

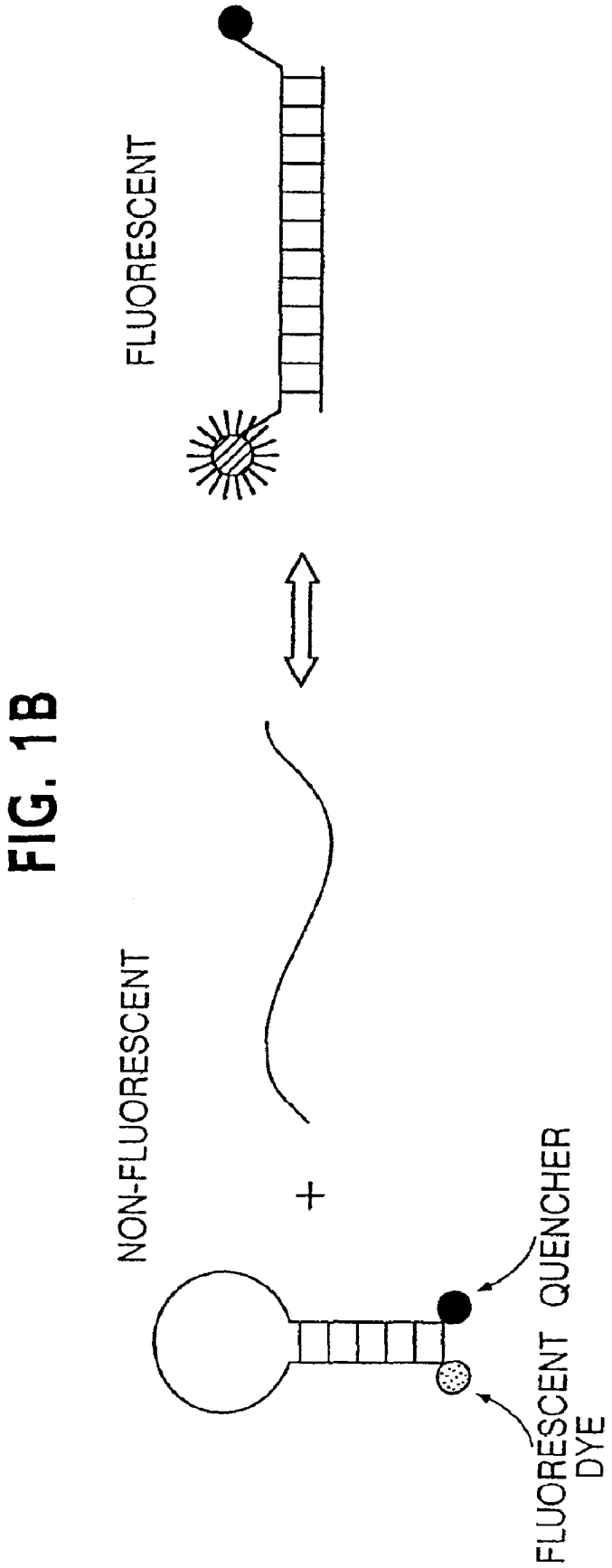


FIG. 1B

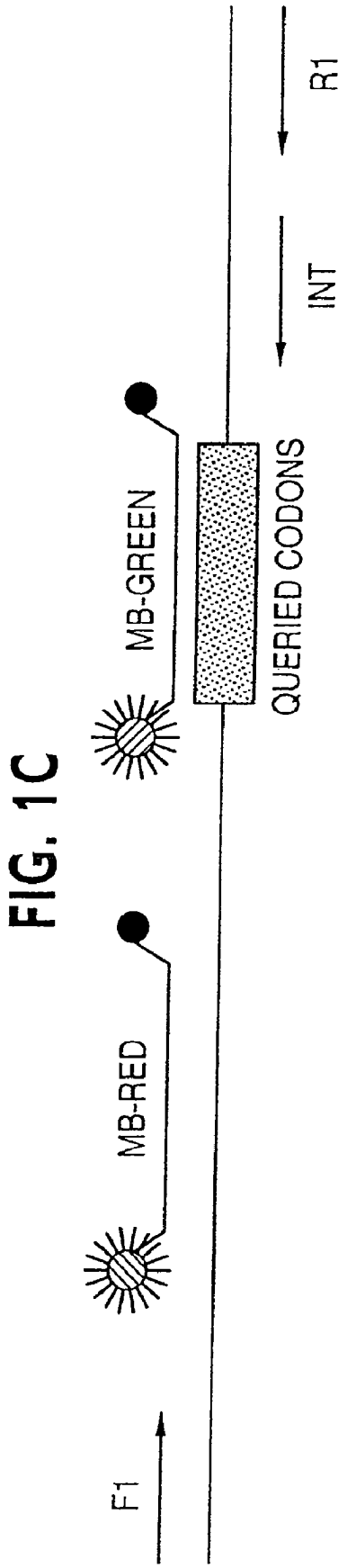


FIG. 1C

FIG. 2

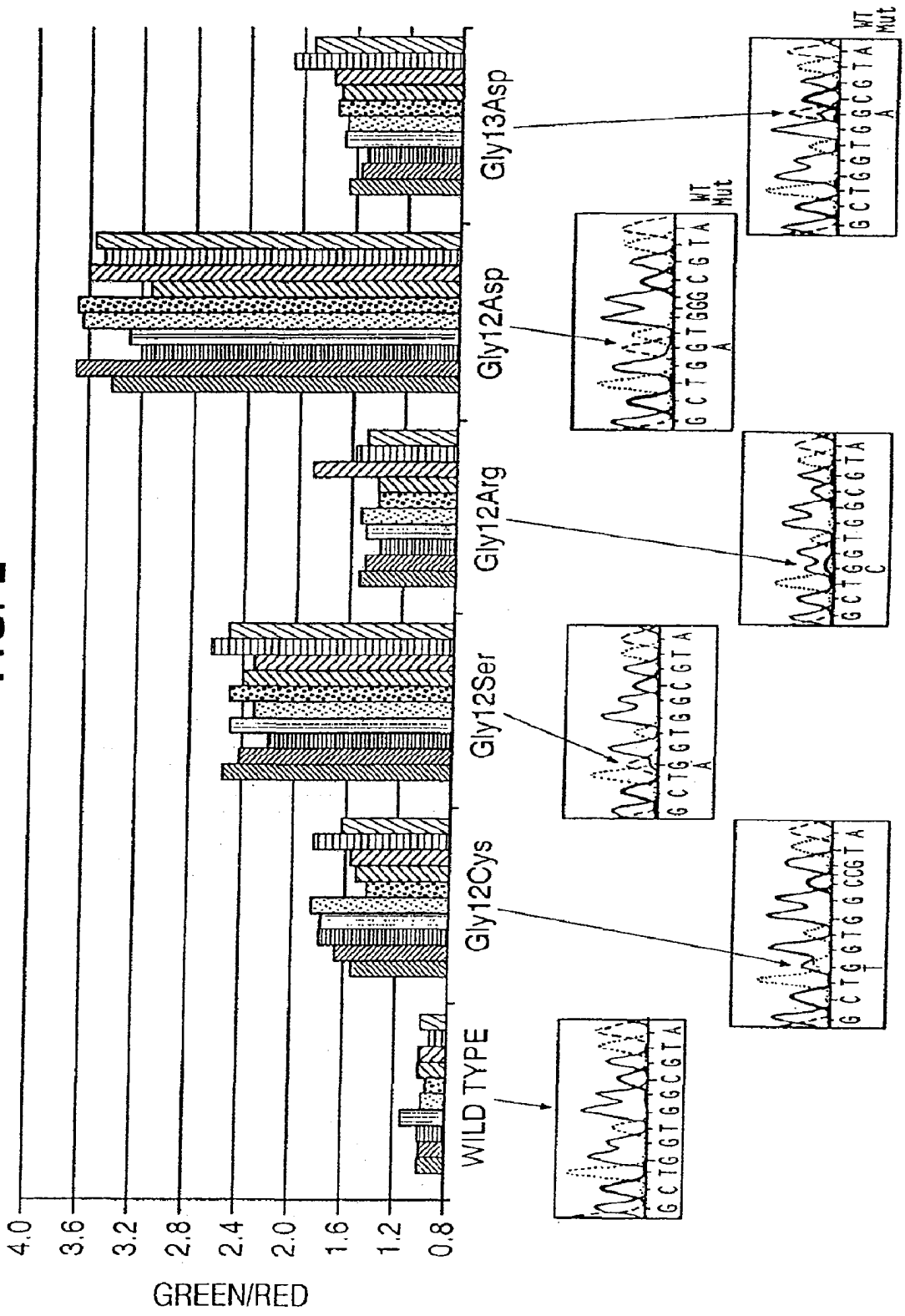
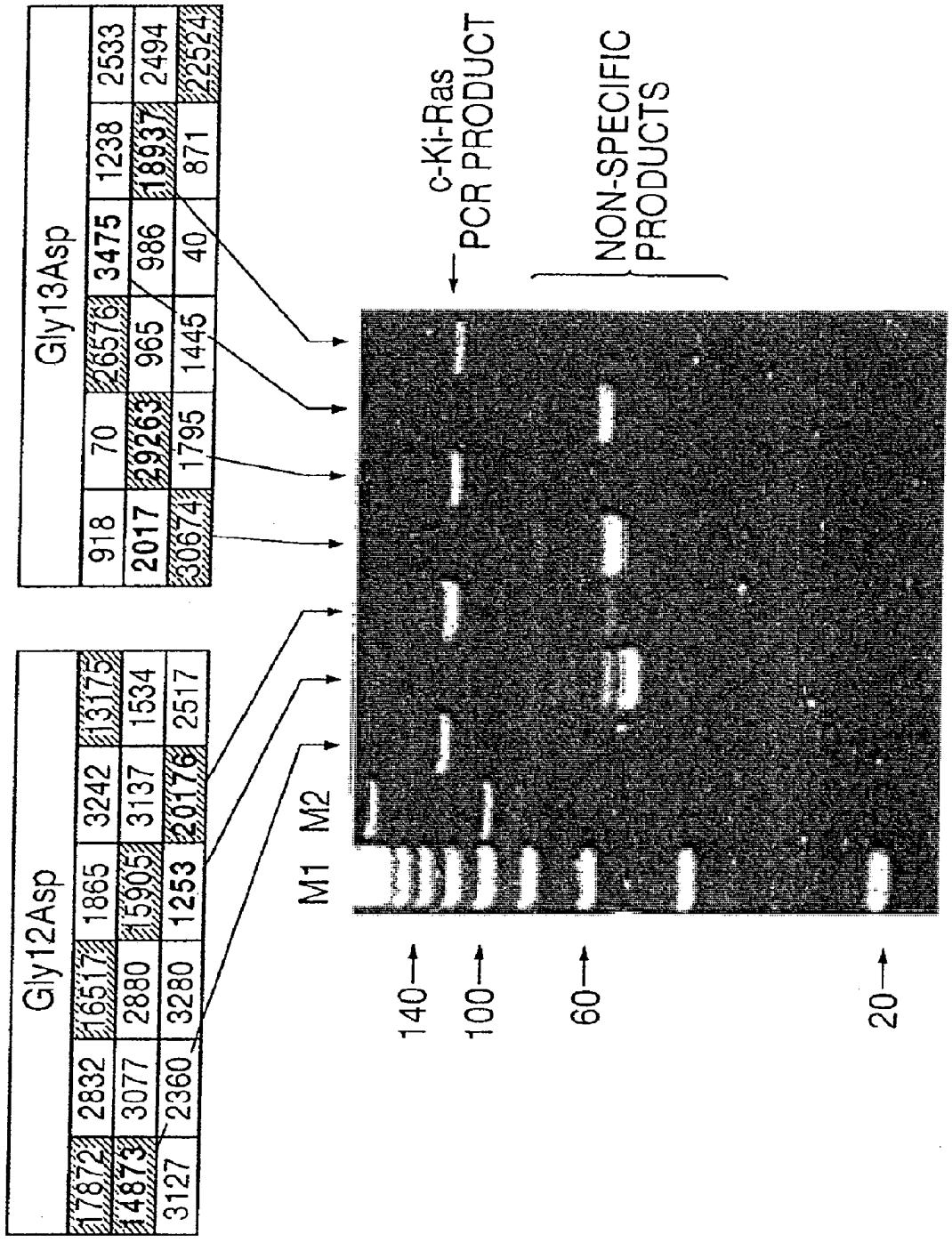

Gly13Asp
Gly12Asp
Gly12Arg
Gly12Ser
Gly12Cys
WILD TYPE
4.0
3.6
3.2
2.8
2.4
2.0
1.6
1.2
0.8
GREEN/RED
WT
Mut

FIG. 3


Gly12Asp
17872
14873
3127
2832
3077
2360
16517
2880
3280
1865
5905
1253
3242
3137
20176
13175
1534
2517
Gly13Asp
918
2017
30674
70
29263
1795
26576
965
1445
3475
986
40
1238
18937
871
2533
2494
22524
M1
M2
c-Ki-Ras
PCR PRODUCT
NON-SPECIFIC
PRODUCTS
140
100
60
20

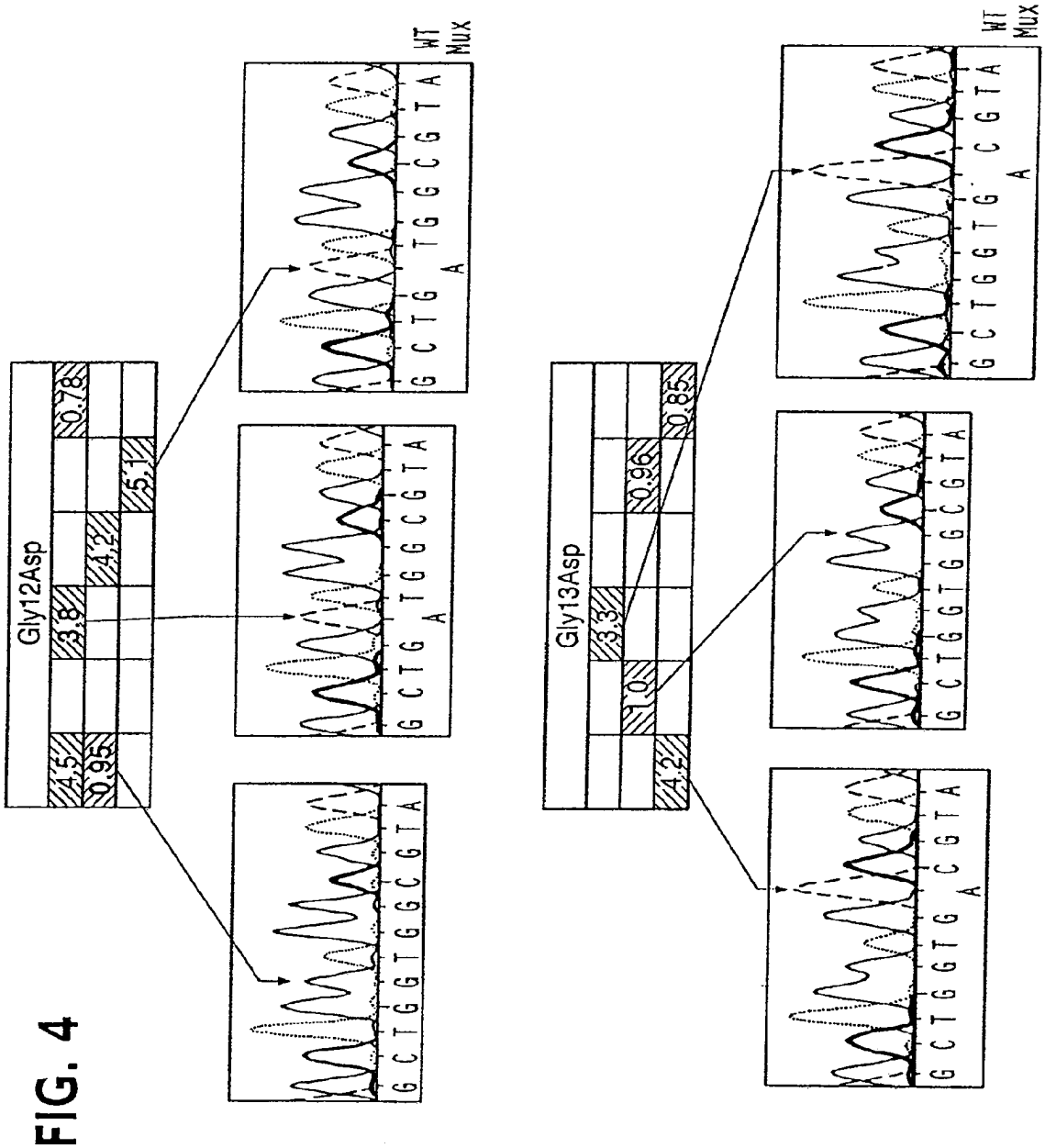
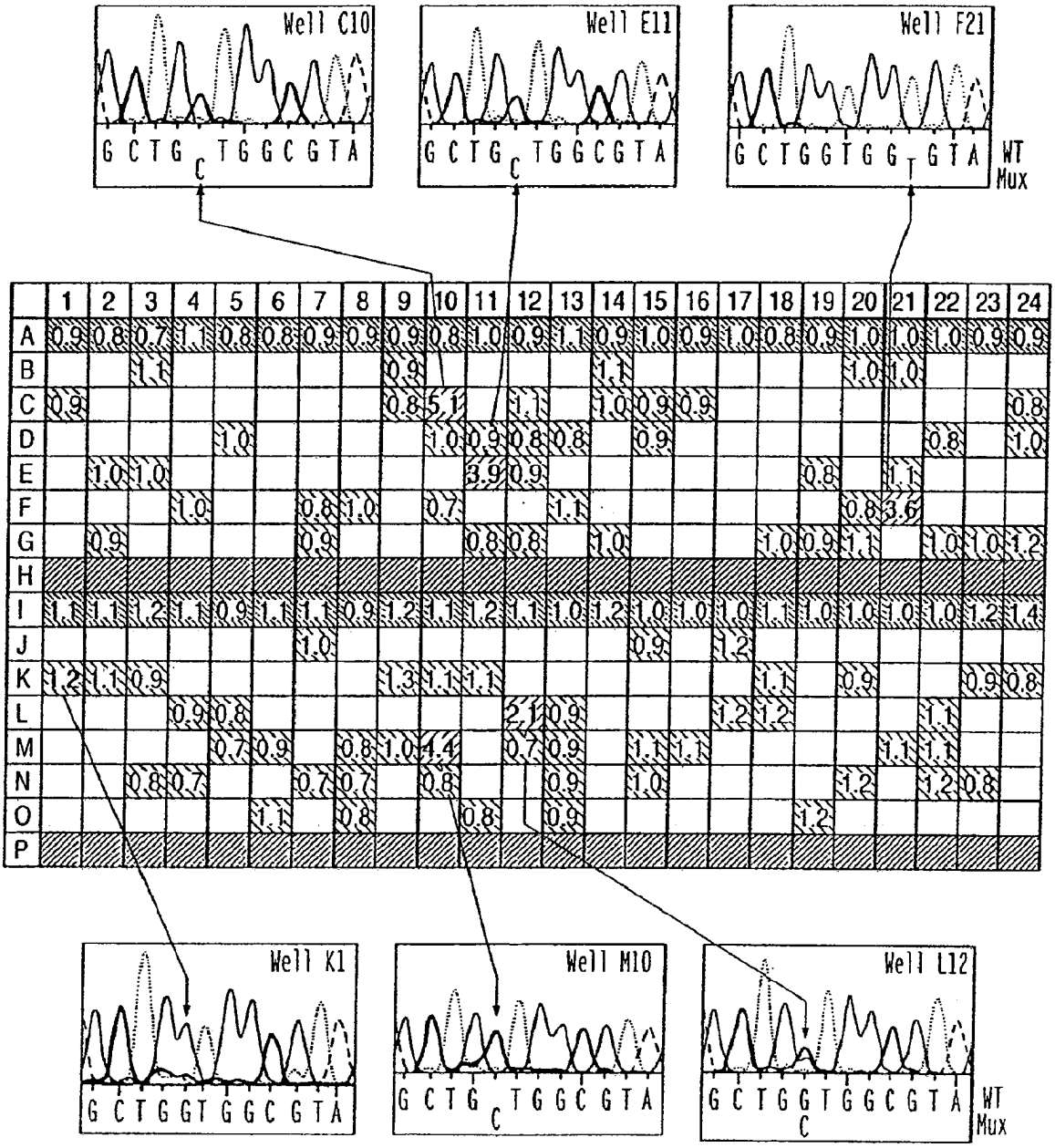

Gly12Asp
0.78
5.1
4.2
3.8
4.5
0.95
WT
MUX
G C T G T G G C G G T A
G C T G T G G C G G T A
G C T G A T G G C G G T A
Gly13Asp
0.85
0.96
3.3
1.0
4.2
WT
MUX
G C T G G T G C G G T A
G C T G G T G C G G T A
G C T G G T G A C G G T A

FIG. 4

FIG. 5


Well C10
G C T G C T G G C G T A
Well E11
G C T G C T G G C G T A
Well F21
G C T G G T G G T G T A
WT
Mux
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20 21 22 23 24
A
B
C
D
E
F
G
H
I
J
K
L
M
N
O
P
Well K1
G C T G G T G G C G T A
Well M10
G C T G C T G G C G T A
Well L12
G C T G G T G G C G T A
WT
Mux

DIGITAL AMPLIFICATION

This application is a division of U.S. application Ser. No. 11/709,742 filed Feb. 23, 2007, which is a continuation of U.S. application Ser. No. 10/828,295 filed Apr. 21, 2004, now abandoned, which is a division of U.S. application Ser. No. 09/981,356 filed Oct. 12, 2001, now U.S. Pat. No. 6,753,147, which is a continuation of U.S. application Ser. No. 09/613,826 filed Jul. 11, 2000, now U.S. Pat. No. 6,440,706, which claims the benefit of provisional U.S. Application Ser. No. 60/146,792, filed Aug. 2, 1999. The disclosure of all priority applications is expressly incorporated herein.

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.

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

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

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

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

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

BRIEF DESCRIPTION OF THE DRAWINGS

FIGS. **1**A, **1**B, **1**C. Schematic of experimental design. (FIG. **1**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. (FIG. **1**B) Principle of molecular beacon analy-

sis. In the stem-loop configuration, fluorescence from a dye at the 5' end of the oligonucleotide probe is quenched by a Dabcyl group at the 3' end. Upon hybridization to a template, the dye is separated from the quencher, resulting in increased fluorescence. Modified from Marras et al. (FIG. **1**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 (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 so that the resultant products have a proportion of the analyte sequence which is detectable by the detection means chosen. At its limit, single template molecules can be amplified so that the products are completely mutant or completely wild-type (WT). The homogeneity of these amplification products makes them trivial to distinguish through existing techniques.

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

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

Digital amplification can be used to detect mutations present at relatively low levels in the samples to be analyzed. The limit of detection is defined by the number of wells that can be analyzed and the intrinsic mutation rate of the polymerase used for amplification. 384 well PCR plates are commercially available and 1536 well plates are on the horizon, theoretically allowing sensitivities for mutation detection at the ~0.1% level. It is also possible that Digital Amplification can be performed in microarray format, potentially increasing the sensitivity by another order of magnitude. This sensitivity may ultimately be limited by polymerase errors. The effective error rate in PCR as performed under our conditions was 1.1%, i.e., four out of 351 PCR products derived from WT DNA sequence appeared to contain a mutation by RED/GREEN ratio criteria. However, any individual mutation (such as a G to T transversion at the second position of codon 12 of c-Ki-Ras), are expected to occur in <1 in 50 of these polymerase-generated mutants (there are at least 50 base substitutions within or surrounding codons 12 and 13 that should yield high RED/GREEN ratios). Determining the sequence of the putative mutants in the positive wells, by direct sequencing as performed here or by any of the other techniques, provides unequivocal validation of a prospective mutation: a significant fraction of the mutations found in individual wells should be identical if the mutation occurred in vivo. Significance can be established through rigorous

statistical analysis, as positive signals should be distributed according to Poisson probabilities. Moreover, the error rate in particular Digital Amplification experiments can be precisely determined through performance of Digital Amplification on DNA templates from normal cells.

Digital Amplification is as easily applied to RT-PCR products generated from RNA templates as it is to genomic DNA. For example, the fraction of alternatively spliced or mutant transcripts from a gene can be easily determined using photoluminescent probes specific for each of the PCR products generated. Similarly, Digital Amplification can be used to quantitate relative levels of gene expression within an RNA population. For this amplification, each well would contain primers which are used to amplify a reference transcript expressed constitutively as well as primers specific for the experimental transcript. One photoluminescent probe would then be used to detect PCR products from the reference transcript and a second photoluminescent probe used for the test transcript. The number of wells in which the test transcript is amplified divided by the number of wells in which the reference transcript is amplified provides a quantitative measure of gene expression. Another group of examples involves the investigations of allelic status when two mutations are observed upon sequence analysis of a standard DNA sample. To distinguish whether one variant is present in each allele (vs. both occurring in one allele), cloning of PCR products is generally performed. The approach described here would simplify the analysis by eliminating the need for cloning. Other potential applications of Digital Amplification are listed in Table 1. When the goal is the quantitation of the proportion of two relatively common alleles or transcripts rather than the detection of rare alleles, techniques such as those employing TaqMan and real time PCR provide an excellent alternative to use of molecular beacons. Advantages of real time PCR methods include their simplicity and the ability to analyze multiple samples simultaneously. However, Digital Amplification may prove useful for these applications when the expected differences are small, (e.g., only ~2-fold, such as occurs with allelic imbalances.)

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 mutant alleles vs. both mutations in same allele	first mutation	second mutation
Allelic Imbalance	Quantitative analysis with non-polymorphic markers	marker sequence	marker from another chromosome

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

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

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

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

The number of different situations in which the digital amplification method will find application is large. Some of these are listed in Table 1. As shown in the examples, the method can be used to find a tumor mutation in a population of cells which is not purely tumor cells. As described in the examples, a probe for a particular mutation need not be used, but diminution in binding to a wild-type probe can be used as an indicator of the presence of one or more mutations. Chromosomal translocations which are characteristic of leukemias or lymphomas can be detected as a measure of the efficacy of therapy. Gene amplifications are characteristic of certain disease states. These can be measured using digital amplification. Alternatively spliced forms of a transcript can be

detected and quantitated relative to other forms of the transcript using digital amplification on cDNA made from mRNA. Similarly, using cDNA made from mRNA one can determine relative levels of transcription of two different genes. One can use digital amplification to distinguish between a situation where one allele carries two mutations and one mutation is carried on each of two alleles in an individual. Allelic imbalances often result from a disease state. These can be detected using digital amplification.

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

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

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

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

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

Example 1

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

Example 2

Step 2: Fluorescence analysis. 3.5 ul of a solution with the following composition was added to each well: 67 mM Tris, pH 8.8, 16.6 mM NH_4SO_4, 6.7 mM $MgCl_2$, 10 mM β-mercaptoethanol, 1 mM dATP, 1 mM dCTP, 1 mM dGTP, 1 mM TTP, 6% DMSO, 5 uM primer INT, 1 uM MB-GREEN, 1 uM MB-RED, 0.1 units/ul Platinum Taq polymerase. The plates were centrifuged for 20 seconds at 6000 g and fluorescence read at excitation/emission wavelengths of 485 nm/530 nm for MB-GREEN and 530 nm/590 nm for MB-RED. This fluorescence in wells without template was typically 10,000 to 20,000 fluorescence "units", with about 75% emanating from the fluorometer background and the remainder from the MB probes. The plates were then placed in a thermal cycler for asymmetric amplification at the following temperatures: 94° for one minute; 10-15 cycles of 94° for 15 sec, 55° for 15 sec., 70° for 15 seconds; 60° for five minutes. The plates were then incubated at room temperature for at least 20 minutes and fluorescence measured as described above. The fluorescence readings obtained were stable for several hours. Specific fluorescence was defined as the difference in fluorescence before and after the asymmetric amplification. RED/GREEN ratios were defined as the specific fluorescence of MB-RED divided by that of MB-GREEN. RED/GREEN ratios were normalized to the ratio exhibited by the positive controls (25 genome equivalents of DNA from normal cells, as defined in Materials and Methods). We found that the ability of MB probes to discriminate between WT and mutant sequences under our conditions could not be reliably determined from experiments in which they were tested by hybridization to relatively short complementary single stranded oligonucleotides, and that actual PCR products had to be used for validation.

Example 3

Oligonucleotides and DNA sequencing. Primer F1: 5'-CATGTTCTAATATAGTC ACATTTTCA-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 synthe-

sized by Gene Link (Thornwood, N.Y.). All were dissolved at 50 uM in TE (10 mM Tris, pH 8.0/1 mM EDTA) and kept frozen and in the dark until use. PCR products were purified using QIAquick PCR purification kits (Qiagen). In the relevant experiments described in the text, 20% of the product from single wells was used for gel electrophoresis and 40% was used for each sequencing reaction. The primer used for sequencing was 5'-CATTATTTTTATTATAAGGCCTGC-3' (SEQ ID NO: 6). Sequencing was performed using fluorescently-labeled ABI Big Dye terminators and an ABI 377 automated sequencer.

Example 4

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

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

A schematic of the oligonucleotides used for Digital Amplifications shown in FIG. **1**C. 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. **1**A, the first step involves amplification from single template molecules. Most protocols for amplification from small numbers of template molecules use a nesting procedure, wherein a product resulting from one set of primers is used as template in a second reaction employing internal primers. As many applications of digital amplification are expected to require hundreds or thousands of separate amplifications, such nesting would be inconvenient and could lead to contamination problems. Hence, conditions were sought that would achieve robust amplification without nesting. The most important of these conditions involved the use of a polymerase that was activated only after heating and optimized concentrations of dNTP's, primers, buffer components, and temperature. The conditions specified in Examples 1-3 were defined after individually optimizing each of these components and proved suitable for amplification of several different human genomic DNA sequences. Though the time required for PCR was not particularly long (~2.5 hr), the number of cycles used was high and excessive compared to the number of cycles required to amplify the "average" single template molecule. The large cycle number was necessary because the template in some wells might not begin to be amplified until several PCR cycles had been completed. The large number of cycles ensured that every well (not simply the average well) would generate a substantial and roughly equal amount of PCR product if a template molecule were present within it.

The second step in FIG. **1**A involves the detection of these PCR products. It was necessary to considerably modify the standard MB probe approach in order for it to function efficiently in Digital Amplification applications. Theoretically, one separate MB probe could be used to detect each specific mutation that might occur within the queried sequence. By inclusion of one MB corresponding to WT sequence and another corresponding to mutant sequence, the nature of the PCR product would be revealed. Though this strategy could obviously be used effectively in some situations, it becomes complex when several different mutations are expected to occur within the same queried sequence. For example, in the c-Ki-Ras gene example explored here, twelve different base substitutions resulting in missense mutations could theoretically occur within codons 12 and 13, and at least seven of these are observed in naturally-occurring human cancers. To detect all twelve mutations as well as the WT sequence with individual Molecular Beacons would require 13 different probes. Inclusion of such a large number of MB probes would not only raise the background fluorescence but would be expensive. We therefore attempted to develop a single probe that would react with WT sequences better than any mutant sequence within the queried sequence. We found that the length of the loop sequence, its melting temperature, and the length and sequence of the stem were each important in determining the efficacy of such probes. Loops ranging from 14 to 26 bases and stems ranging from 4 to 6 bases, as well as numerous sequence variations of both stems and loops, were tested during the optimization procedure. For discrimination between WT and mutant sequences (MB-GREEN probe), we found that a 16 base pair loop, of melting temperature (Tm) 50-51°, and a 4 bp stem, of sequence 5'-CACG-3', were optimal. For MB-RED probes, the same stem, with a 19-20 bp loop of Tm 54-56°, proved optimal. The differences in the loop sizes and melting temperatures between MB-GREEN and MB-RED probes reflected the fact that only the GREEN probe is designed to discriminate between closely related sequences, with a shorter region of homology facilitating such discrimination.

Examples of the ratios obtained in replicate wells containing DNA templates from colorectal tumor cells with mutations of c-Ki-Ras are shown in FIG. **2**. In this experiment, fifty copies of genomic DNA equivalents were diluted into each well prior to amplification. Each of six tested mutants yielded ratios of RED/GREEN fluorescence that were significantly in excess of the ratio obtained with DNA from normal cells (1.5 to 3.4 in the mutants compared to 1.0 in normal DNA; $p<0.0001$ in each case, Student's t-Test). The reproducibility

of the ratios can be observed in this figure. Direct DNA sequencing of the PCR products used for fluorescence analysis showed that the RED/GREEN ratios were dependent on the relative fraction of mutant genes within the template population (FIG. **2**). Thus, the DNA from cells containing one mutant C-Ki-Ras allele per every two WT c-Ki-Ras allele yielded a RED/GREEN ratio of 1.5 (Gly12Arg mutation) while the cells containing three mutant c-Ki-Ras alleles per WT allele exhibited a ratio of 3.4 (Gly12Asp). These data suggested that wells containing only mutant alleles (no WT) would yield ratios in excess of 3.0, with the exact value dependent on the specific mutation.

Though this mode is the most convenient for many applications, we found it useful to add the MB probes after the PCR-amplification was complete (FIG. **1**). This allowed us to use a standard multiwell plate fluorometer to sequentially analyze a large number of multiwell plates containing preformed 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. **1**C) 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. **5**B). 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).

SEQUENCE LISTING

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The invention claimed is:

1. A method for determining an allelic imbalance in 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 the biological sample;

analyzing the amplified molecules in the assay samples of the set to determine a first number of assay samples which contain a first allelic form of a marker and a second number of assay samples which contain a second allelic form of the marker, wherein between 0.1 and 0.9 of the assay samples yield an amplification product;

comparing the first number to the second number to ascertain an allelic imbalance in the biological sample; and

identifying an allelic imbalance in the biological sample.

2. The method of claim **1** wherein the step of amplifying employs real-time polymerase chain reactions.

3. The method of claim **2** wherein the real-time polymerase chain reactions comprise a dual-labeled fluorogenic probe.

4. The method of claim **1** wherein between 0.1 and 0.9 of the assay samples yield an amplification product as determined by amplification of the first allelic form of the marker.

5. The method of claim **1** wherein between 0.1 and 0.9 of the assay samples yield an amplification product as determined by amplification of the second allelic form of the marker.

6. The method of claim **1** wherein the amplified molecules in each of the assay samples within the first and second numbers of assay samples are homogeneous such that the first number of assay samples do not contain the second allelic form of the marker and the second number of assay samples do not contain the first allelic form of the marker.

7. The method of claim **1** wherein the sample is from blood.

8. A method for determining an allelic imbalance in a biological sample, comprising the steps of:

distributing nucleic acid template molecules from 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 a first allelic form of a marker and a second number of assay samples which contain a second allelic form of the marker;

comparing the first number of assay samples to the second number of assay samples to ascertain an allelic imbalance between the first allelic form and the second allelic form in the biological sample.

9. The method of claim **8** wherein the sample is from blood.

10. The method of claim **1** or **8** wherein between 0.1 and 0.6 of the assay samples yield an amplification product.

11. The method of claim **1** or **8** wherein between 0.3 and 0.5 of the assay samples yield an amplification product.

12. The method of claim **1** or **8** wherein the set comprises at least 500 assay samples.

13. The method of claim **1** or **8** wherein the set comprises at least 1000 assay samples.

14. The method of claim **8** wherein the step of amplifying employs real-time polymerase chain reactions.

15. The method of claim **14** wherein the real-time polymerase chain reactions comprise a dual-labeled fluorogenic probe.

16. The method of claim **8** wherein between 0.1 and 0.9 of the assay samples yield an amplification product as determined by amplification of the first allelic form of the marker.

17. The method of claim **8** wherein between 0.1 and 0.9 of the assay samples yield an amplification product as determined by amplification of the second allelic form of the marker.

18. The method of claim **8** wherein the amplified molecules in each of the assay samples within the first and second numbers of assay samples are homogeneous such that the first number of assay samples do not contain the second allelic form of the marker and the second number of assay samples do not contain the first allelic form of the marker.

* * * * *

EXHIBIT 11

PATENT

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

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

RESPONSE TO OFFICE ACITON

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

Sir:

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

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

IN THE CLAIMS

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

1. -48. (Cancelled)

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

Remarks

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

The rejection under § 112, second paragraph

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

Rejection under § 102(b)

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

Rejection under § 102(a)

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

The first rejection under § 103(a)

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

The second rejection under § 103(a)

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

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

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

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

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

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

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

Respectfully submitted,

Date: *March 11, 2013*

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

Banner & Witcoff, Ltd.
Customer No. 11332

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

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

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

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

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

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

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

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

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

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

WARNING: Information on this form may become public. Credit card information should not be included on this form. Provide credit card information and authorization on PTO-2038.

I am the

☐ applicant/inventor.

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

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

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

/Sarah A. Kagan/	11 March 2013
Signature	Date
Sarah A. Kagan	(202) 824-3000
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.

☑ Total of 1 forms are submitted.

Privacy Act Statement

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

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

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

Electronic Patent Application Fee Transmittal

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

Filed as Large Entity

Utility under 35 USC 111(a) Filing Fees

Description	Fee Code	Quantity	Amount	Sub-Total in USD($)
Basic Filing:				
Pages:				
Claims:				
Miscellaneous-Filing:				
Petition:				
Patent-Appeals-and-Interference:				
Post-Allowance-and-Post-Issuance:				
Extension-of-Time:				
Extension - 2 months with $0 paid	1252	1	570	570

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

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

Payment information:

Submitted with Payment	yes
Payment Type	Deposit Account
Payment was successfully received in RAM	$570
RAM confirmation Number	22590
Deposit Account	190733
Authorized User	
The Director of the USPTO is hereby authorized to charge indicated fees and credit any overpayment as follows: Charge any Additional Fees required under 37 C.F.R. Section 1.16 (National application filing, search, and examination fees) Charge any Additional Fees required under 37 C.F.R. Section 1.17 (Patent application and reexamination processing fees)	

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

File Listing:

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

Multipart Description/PDF files in .zip description

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

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

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

New Applications Under 35 U.S.C. 111

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

National Stage of an International Application under 35 U.S.C. 371

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

New International Application Filed with the USPTO as a Receiving Office

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

PTO/SB/06 (07-06)
Approved for use through 1/31/2007. OMB 0651-0032
U.S. Patent and Trademark Office; U.S. DEPARTMENT OF COMMERCE
Under the Paperwork Reduction Act of 1995, no persons are required to respond to a collection of information unless it displays a valid OMB control number.

PATENT APPLICATION FEE DETERMINATION RECORD

Substitute for Form PTO-875

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

APPLICATION AS FILED – PART I

	(Column 1)	(Column 2)	SMALL ENTITY ☐		OR	OTHER THAN SMALL ENTITY	
FOR	NUMBER FILED	NUMBER EXTRA	RATE ($)	FEE ($)		RATE ($)	FEE ($)
☐ BASIC FEE (37 CFR 1.16(a), (b), or (c))	N/A	N/A	N/A			N/A	
☐ SEARCH FEE (37 CFR 1.16(k), (i), or (m))	N/A	N/A	N/A			N/A	
☐ EXAMINATION FEE (37 CFR 1.16(o), (p), or (q))	N/A	N/A	N/A			N/A	
TOTAL CLAIMS (37 CFR 1.16(i))	minus 20 =	*	X $ =		OR	X $ =	
INDEPENDENT CLAIMS (37 CFR 1.16(h))	minus 3 =	*	X $ =			X $ =	
☐ APPLICATION SIZE FEE (37 CFR 1.16(s))	If the specification and drawings exceed 100 sheets of paper, the application size fee due is $250 ($125 for small entity) for each additional 50 sheets or fraction thereof. See 35 U.S.C. 41(a)(1)(G) and 37 CFR 1.16(s).						
☐ MULTIPLE DEPENDENT CLAIM PRESENT (37 CFR 1.16(j))							
* If the difference in column 1 is less than zero, enter "0" in column 2.			TOTAL			TOTAL	

APPLICATION AS AMENDED – PART II

AMENDMENT	03/11/2013	(Column 1) CLAIMS REMAINING AFTER AMENDMENT		(Column 2) HIGHEST NUMBER PREVIOUSLY PAID FOR	(Column 3) PRESENT EXTRA	SMALL ENTITY RATE ($)	ADDITIONAL FEE ($)	OR	OTHER THAN SMALL ENTITY RATE ($)	ADDITIONAL FEE ($)
	Total (37 CFR 1.16(i))	* 20	Minus	** 68	= 0	X $ =		OR	X $62=	0
	Independent (37 CFR 1.16(h))	* 1	Minus	***6	= 0	X $ =		OR	X $250=	0
	☐ Application Size Fee (37 CFR 1.16(s))									
	☐ FIRST PRESENTATION OF MULTIPLE DEPENDENT CLAIM (37 CFR 1.16(j))							OR		
						TOTAL ADD'L FEE		OR	TOTAL ADD'L FEE	0

AMENDMENT		(Column 1) CLAIMS REMAINING AFTER AMENDMENT		(Column 2) HIGHEST NUMBER PREVIOUSLY PAID FOR	(Column 3) PRESENT EXTRA	RATE ($)	ADDITIONAL FEE ($)		RATE ($)	ADDITIONAL FEE ($)
	Total (37 CFR 1.16(i))	*	Minus	**	=	X $ =		OR	X $ =	
	Independent (37 CFR 1.16(h))	*	Minus	***	=	X $ =		OR	X $ =	
	☐ Application Size Fee (37 CFR 1.16(s))									
	☐ FIRST PRESENTATION OF MULTIPLE DEPENDENT CLAIM (37 CFR 1.16(j))							OR		
						TOTAL ADD'L FEE		OR	TOTAL ADD'L FEE	

* If the entry in column 1 is less than the entry in column 2, write "0" in column 3.

** If the "Highest Number Previously Paid For" IN THIS SPACE is less than 20, enter "20".

*** If the "Highest Number Previously Paid For" IN THIS SPACE is less than 3, enter "3".

The "Highest Number Previously Paid For" (Total or Independent) is the highest number found in the appropriate box in column 1.

Legal Instrument Examiner:
/SHARON HARRIS/

UNITED STATES PATENT AND TRADEMARK OFFICE

UNITED STATES DEPARTMENT OF COMMERCE
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www.uspto.gov

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

11332 7590 10/10/2012

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EXAMINER	
WOOLWINE, SAMUEL C	
ART UNIT	PAPER NUMBER
1637	

MAIL DATE	DELIVERY MODE
10/10/2012	PAPER

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

The time period for reply, if any, is set in the attached communication.

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

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

Period for Reply

A SHORTENED STATUTORY PERIOD FOR REPLY IS SET TO EXPIRE 3 MONTH(S) OR THIRTY (30) DAYS, WHICHEVER IS LONGER, FROM THE MAILING DATE OF THIS COMMUNICATION.

- Extensions of time may be available under the provisions of 37 CFR 1.136(a). In no event, however, may a reply be timely filed after SIX (6) MONTHS from the mailing date of this communication.
- If NO period for reply is specified above, the maximum statutory period will apply and will expire SIX (6) MONTHS from the mailing date of this communication.
- Failure to reply within the set or extended period for reply will, by statute, cause the application to become ABANDONED (35 U.S.C. § 133). Any reply received by the Office later than three months after the mailing date of this communication, even if timely filed, may reduce any earned patent term adjustment. See 37 CFR 1.704(b).

Status

1)☒ Responsive to communication(s) filed on *11 June 2012*.

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

3)☐ An election was made by the applicant in response to a restriction requirement set forth during the interview on _____; the restriction requirement and election have been incorporated into this action.

4)☐ Since this application is in condition for allowance except for formal matters, prosecution as to the merits is closed in accordance with the practice under *Ex parte Quayle*, 1935 C.D. 11, 453 O.G. 213.

Disposition of Claims

5)☒ Claim(s) *1-68* is/are pending in the application.

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

6)☐ Claim(s) _____ is/are allowed.

7)☒ Claim(s) *49-68* is/are rejected.

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

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

Application Papers

10)☐ The specification is objected to by the Examiner.

11)☐ The drawing(s) filed on _____ is/are: a)☐ accepted or b)☐ objected to by the Examiner.

Applicant may not request that any objection to the drawing(s) be held in abeyance. See 37 CFR 1.85(a).

Replacement drawing sheet(s) including the correction is required if the drawing(s) is objected to. See 37 CFR 1.121(d).

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

Priority under 35 U.S.C. § 119

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

a)☐ All b)☐ Some * c)☐ None of:

1.☐ Certified copies of the priority documents have been received.

2.☐ Certified copies of the priority documents have been received in Application No. _____.

3.☐ Copies of the certified copies of the priority documents have been received in this National Stage application from the International Bureau (PCT Rule 17.2(a)).

* See the attached detailed Office action for a list of the certified copies not received.

Attachment(s)

1) ☒ Notice of References Cited (PTO-892)
2) ☐ Notice of Draftsperson's Patent Drawing Review (PTO-948)
3) ☒ Information Disclosure Statement(s) (PTO/SB/08) Paper No(s)/Mail Date *03/24/2011*.
4) ☐ Interview Summary (PTO-413) Paper No(s)/Mail Date. _____ .
5) ☐ Notice of Informal Patent Application
6) ☐ Other: _____.

DETAILED ACTION

Election/Restrictions

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

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

Claim Rejections - 35 USC § 112

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

> The specification shall conclude with one or more claims particularly pointing out and distinctly claiming the subject matter which the applicant regards as his invention.

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

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

Claim Rejections - 35 USC § 102

The following is a quotation of the appropriate paragraphs of 35 U.S.C. 102 that form the basis for the rejections under this section made in this Office action:

A person shall be entitled to a patent unless –

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

(b) the invention was patented or described in a printed publication in this or a foreign country or in public use or on sale in this country, more than one year prior to the date of application for patent in the United States.

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

With regard to claim 49, Li taught:

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

Claim Rejections - 35 USC § 103

The following is a quotation of 35 U.S.C. 103(a) which forms the basis for all obviousness rejections set forth in this Office action:

> (a) A patent may not be obtained though the invention is not identically disclosed or described as set forth in section 102 of this title, if the differences between the subject matter sought to be patented and the prior art are such that the subject matter as a whole would have been obvious at the time the invention was made to a person having ordinary skill in the art to which said subject matter pertains. Patentability shall not be negatived by the manner in which the invention was made.

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

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

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

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

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

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

The teachings of Irving have been discussed.

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

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

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

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

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

Conclusion

No claims are free of the prior art.

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

If attempts to reach the examiner by telephone are unsuccessful, the examiner's supervisor, Gary Benzion can be reached on (571) 272-0782. The

fax phone number for the organization where this application or proceeding is assigned is 571-273-8300.

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

/Samuel Woolwine/
Primary Examiner

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

U.S. PATENT DOCUMENTS

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

*		Include as applicable: Author, Title Date, Publisher, Edition or Volume, Pertinent Pages)
	U	Irving et al. TT Virus Infection in Patients with Hepatitis C: Frequency, Persistence, and Sequence Heterogeneity. The Journal of Infectious Diseases 180:27-34, July 1999.
	V	Simmonds et al. Human immunodeficiency virus-infected individuals contain provirus in small numbers of peripheral mononuclear cells and at low copy numbers. Journal of Virology 64(2):864-872 (1990).
	W	
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Filing Date	2011-03-16
First Named Inventor	Bert Vogelstein et al.
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	3	5518901	A	1996-05-21	Murtagh	
	4	5804383	A	1998-09-08	Gruenert et al.	
	5	5858663	A	1999-01-12	Nisson et al.	
	6	5670325	A	1997-09-23	Lapidus et al.	
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	9	5928870	A	1999-07-27	Lapidus et al.	
	10	6020137	A	2000-02-01	Lapidus et al.	
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	1	LOUGHLIN ET AL., "Association of the Interleukin-1 Gene Cluster on Chromosome 2q13 With Knee Osteoarthritis," Arthritis & Rheumatism, June 2002, 46(6):1519-1527	☐
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	3	A. PIATEK ET AL., "Molecular Beacon Sequence Analysis for Detecting Drug Resistance in Mycobacterium Tuberculosis," Nature Biotechnology, April 1998, Vol. 16, No. 4. pp. 359-363	☐
	4	S. TYAGI ET AL., "Multicolor Molecular Beacons for allele discrimination," Nature Biotechnology, January 1998, Vol. 16, No. 1, pp. 303-308	☐
	5	J. A.M. VET ET AL., "Multilex Detection of Four Pathogenic Retroviruses Using Molecular Beacons," Proceedings of the National Academy of Sciences of the United States", May 25, 1999, Vol. 96, No. 11, pp. 6394-6399	☐
	6	S. TYAGI ET AL., "Molecular Beacons: probes that Fluoresce Upon Hybridization," Nature Biotechnology, 1996, Vol. 14, No. 3, pp. 303-308	☐
	7	W. P. HALFORD ET AL., "The Inherent Quantitative Capacity of the Reverse Transcription-Polymerase Chain Reaction," Analytical Biochemistry, January 15, 1999, Vol. 266, No. 2, pp. 181-191	☐
	8	B. VOGELSTEIN ET AL., "Digital PCR," Proceedings of the National Academy of Sciences of the United States, August 3, 1999, Vol. 96, No. 16, pp. 9236-9241	☐

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

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	20	MARRAS ET AL., "Multiplex Detection of Single-Nucleotide Variations Using Molecular Beacons," Genetic Analysis: Biomolecular Engineering, Feb. 1999, Vo. 14, pp. 151-156	☐
	21	WHITCOMB ET AL., "Detection of PCR Products Using Self-Probing Amplicons and Fluorescence," Nature Biotechnology, August 1999, Vol. 17, pp. 804-807	☐
	22	M.J. BRISCO ET AL., "Detection and Quantitation of Neoplastic Cells in Acute Lymphoblastic Leukemia, by Use of the Polymerase Chain Reaction," British Journal of Haematology, 1991, Vol. 79, pp. 211-217	☐
	23	M. J. BRISCO ET AL., "Outcome Prediction in Childhood Acute Lymphoblastic Leukemia by Molecular Quantification of Residual Disease at the End of Induction," The Lancet, January 22, 1994, Vol. 343, pp. 196-200	☐
	24	Notice of Reasons for Rejection dispatched April 28, 2010 in Japanese Application No. 2001-513641 and English translation thereof.	☐
	25	Stephens, J. Clairborne, et al. "Theoretical underpinning of the Single-Molecular-Dilution (SMD) Method of Direct Haplotype Resolution," Am. J. Hum. Gen., Vol. 46, pp. 1149-1155 (1990).	☐
	26	NEWTON, PCR Essential Data, pages 51-52, 1995	☐
	27	Office Action dated June 11, 2010, in co-pending application 11/709,742	☐
	28	Office Action dated December 29, 2009 in co-pending application 11/709,742	☐
	29	Office Action dated September 18, 2009 in co-pending application 11/709,742	☐
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L1	2449	(sample with (split splitting divide divided dividing dilute diluting diluted dilution)) same ((fragment molecule template) near5 (single one "less than" "more than" "greater than" "fewer than" fewer less))	US-PGPUB; USPAT; USOCR; FPRS; EPO; JPO; DERWENT; IBM_TDB	OR	ON	2012/10/02 15:01
L2	361	l1 and (@ad<"19990802" @pd<"19990802")	US-PGPUB; USPAT; USOCR; FPRS; EPO; JPO; DERWENT; IBM_TDB	OR	ON	2012/10/02 15:01
L3	12232	rare near5 (sequence target mutation variant variation polymorphism)	US-PGPUB; USPAT; USOCR; FPRS; EPO; JPO; DERWENT; IBM_TDB	OR	ON	2012/10/02 15:02
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S2	4	("7915015" "7824889" "6753147" "6440706").PN.	USPAT	OR	OFF	2012/10/01 07:49
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			JPO; DERWENT; IBM_TDB			
S7	26	S5 and (sequencing sequenced) with ((pcr amplification) near2 product)	US-PGPUB; USPAT; USOCR; FPRS; EPO; JPO; DERWENT; IBM_TDB	OR	ON	2012/10/01 17:05
S8	4	(vogelstein kinzler).in. and (dilut$3 distribut$3).clm. and (sequencing (determin$5 near2 sequence)).clm. and (samples aliquots portions tubes wells).clm.	US-PGPUB; USPAT; USOCR; FPRS; EPO; JPO; DERWENT; IBM_TDB	OR	ON	2012/10/01 17:13
S9	289	((distributing diluting aliquotting dividing splitting) with (dna nucleic sample)) same (amplif$7 pcr) same (sequencing sequenced)	US-PGPUB; USPAT; USOCR; FPRS; EPO; JPO; DERWENT; IBM_TDB	OR	ON	2012/10/01 17:31
S10	16	S9 and (@ad<"19990802" @pd<"19990802")	US-PGPUB; USPAT; USOCR; FPRS; EPO; JPO; DERWENT; IBM_TDB	OR	OFF	2012/10/01 17:31
S11	1337	((distributing diluting aliquotting dividing splitting) with (dna nucleic sample)) same (amplif$7 pcr) and (sequencing sequenced)	US-PGPUB; USPAT; USOCR; FPRS; EPO; JPO; DERWENT; IBM_TDB	OR	ON	2012/10/01 17:32
S12	312	S11 and ((less fewer) near5 molecules)	US-PGPUB; USPAT; USOCR; FPRS; EPO; JPO; DERWENT; IBM_TDB	OR	ON	2012/10/01 17:33
S13	8	S12 and (@ad<"19990802" @pd<"19990802")	US-PGPUB; USPAT; USOCR; FPRS; EPO; JPO; DERWENT; IBM_TDB	OR	OFF	2012/10/01 17:33
S18	506	(rare adj1 (sequence target mutation)) and ((pcr "polymerase chain") same (dilution diluting diluted))	US-PGPUB; USPAT; USOCR; FPRS; EPO; JPO; DERWENT; IBM_TDB	OR	ON	2012/10/01 22:55
S19	56	S18 and (@ad<"19990802" @pd<"19990802")	US-PGPUB; USPAT;	OR	ON	2012/10/01 22:55

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

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EAST Search History

EAST Search History (Prior Art)

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

10/ 2/ 2012 5:51:13 PM
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