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 3

Date: June 17, 2013

1. [✔] This is a request for *ex parte* reexamination pursuant to 37 CFR 1.510 of patent number 7,915,015 issued March 29, 2011. 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) 1-18 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 & 7824889

[ ] 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. 7,915,015	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 1-18 of U.S. Patent No. 7,915,015 entitled "DIGITAL AMPLIFICATION" ("the '015 patent"). The '015 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. 7,915,015 to Vogelstein et al., titled "Digital Amplification," issued on March 29, 2011, with a priority date of August 2, 1999 and terminal disclaimer filed October 6, 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: Li *et al.*, Nature. 29;335(6189):414-7 (Sep 29, 1988)

Exhibit PA-4: 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. 7,915,015

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 1-18 of U.S. Patent No. 7,915,015 to Vogelstein *et al.* ("the '015 patent"), and currently assigned to The Johns Hopkins University. The '015 patent issued on November 2, 2010, with a priority date of August 2, 1999.

Reexamination of claims 1-18 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	**"LI"** Li *et al.*, Nature. 29;335(6189):414-7 (Sep 29, 1988)	**102(B)/ 103**	**YES**	**NO**
PA-4	**"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, 4, 5, 7-11 & 16-17 under 35 U.S.C. § 102(b)
SNQ No. 2:	Claims 2, 3, 14 & 15 of the '015 patent are obvious under 35 U.S.C. § 103(a) over Bischoff in view of Kalinina
SNQ No. 3:	Claims 12 & 13 of the '015 patent are obvious under 35 U.S.C. § 103(a) over Bischoff in view of Li
SNQ No. 4:	Claims 6 & 18 of the '015 patent are obvious under 35 U.S.C. § 103(a) over Bischoff in view of Ruano II

Table III **Proposed Rejections**	
Proposed Rejection No. 1:	Bischoff anticipates claims 1, 4, 5, 7-11 & 16-17 under

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

	35 U.S.C. § 102(b)
Proposed Rejection No. 2:	Claims 2, 3, 14 & 15 of the '015 patent are obvious under 35 U.S.C. § 103(a) over Bischoff in view of Kalinina
Proposed Rejection No. 3:	Claims 12 & 13 of the '015 patent are obvious under 35 U.S.C. § 103(a) over Bischoff in view of Li
Proposed Rejection No. 4:	Claims 6 & 18 of the '015 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 '015 PATENT ARE GIVEN THEIR BROADEST REASONABLE INTERPRETATION IN REEXAMINATION, UNLIKE THE STANDARDS APPLICABLE IN THE CONCURRENT LITIGATION

The '015 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 '015 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 and all claim interpretations discussed or submitted herein, and all applications of the prior art to the claims, are under the broadest reasonable interpretation specifically for the purpose of demonstrating a SNQ for reexamination within the PTO and are neither binding upon Requester in any litigation related to the '015 patent, nor necessarily the construction of the claims that would result 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); *see also Trans Texas,* 498 F.3d at 1297-98; *In re Zletz*, 893 F.2d 319, 322 (Fed. Cir. 1989).

The interpretation and/or construction of the claims in the '015 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. 7,915,015 (the '015 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 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.

IV. PROSECUTION HISTORY OF THE '015 AND PARENT '706 PATENTS

During prosecution of the '015 patent, no prior art was applied against the '015 claims (except for the claims of the grandparent 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 '015 claims, and either anticipate or render these claims obvious.

No art was applied against the '015 claims during original prosecution, or against the claims of its immediate parent, now U.S. Pat. No. 7,824,889 But art was applied against the claims of a grandparent patent (U.S. 6,440,706, hereafter the '706 patent, for which Requester is concurrently requesting reexamination). For the purposes of patentability in this reexamination, the '706 claims were substantially similar to the '015 claims. Generally speaking, claims of both the '706 and '015 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 contain one sequence and a second number of assay samples that contain 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

[2] Prosecution history of the '015 patent, Office Action mailed September 23, 2010, at page 5 (Exhibit 3).

reflects the composition of the biological sample, whereas the '015 claims generally require that the comparing is performed to ascertain an allelic imbalance.

During original prosecution of the '706 claims, the PTO rejected multiple claims of 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 selected '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 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

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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)

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

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 during prosecution of the grandparent '706 patent (*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 '015 patent for which reexamination is sought. *Ex parte* reexamination of claims 1-18 of the '015 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.

A. SNQ No. 1: Bischoff anticipates claims 1, 4, 5, 7-11 & 16-17 under 35 U.S.C. § 102(b)

Bischoff[8] was published in March 1995 and is thus prior art to the '015 patent under 35 U.S.C. § 102(b). Bischoff is newly cited in the present request. Under the

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

broadest reasonable interpretation of the claims, Bischoff discloses methods that meet all of the limitations of the methods of claims 1, 4, 5, 7-11 & 16-17.

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, 4, 5, 7-11 & 16-17 and squarely anticipates these claims. In contrast, during the original prosecution of the '015 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, 4, 5, 7-11 & 16-17.

B. SNQ No. 2: Claims 2, 3, 14 and 15 of the '015 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 '015 patent under 35 U.S.C. § 102(b). Kalinina is newly cited in the present request.

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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 '015 patent under 35 U.S.C. § 102(b) (Exhibit PA-1).

[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 '015 patent under 35 U.S.C. § 102(b) (Exhibit PA-2).

Bischoff and Kalinina together raise a new question of patentability as to claims 2, 3, 14 and 15 because they were neither cited nor considered during the prosecution of the '015 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, 3, 14 and 15 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, 3, 14 and 15.

C. SNQ No. 3: Claims 12 & 13 of the '015 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[10] was published on September 29, 1988 and is prior art to the '015 patent under 35 U.S.C. § 102(b). Although cited by the applicants, Li was not relied on or discussed on record 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 examiner would also have rejected the claims of the' 889 patent, which are similar to the rejected claims of the '105 application.

[10] 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 '015 patent under 35 U.S.C. § 102(b) (Exhibit PA-4).

Bischoff and Li together raise a new question of patentability as to claims 12 & 13 at least because Bischoff was neither cited nor considered during the prosecution of the '015 patent. Also, Li was not specifically discussed 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 12 & 13 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 12 & 13.

D. SNQ No. 4: Claims 6 & 18 of the '015 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[11] was published on October 25, 1989 and is prior art to the '015 patent under 35 U.S.C. § 102(b). Ruano II is newly cited in the present request.

Bischoff and Ruano II together raise a new question of patentability as to claims 6 & 18 because they were neither cited nor considered during the prosecution of the '015 patent.

[11] Ruano *et al.*, Nucleic Acids Res. 17(20):8392 (Oct 25, 1989), which forms prior art to the '015 patent under 35 U.S.C. § 102(b) (Exhibit PA-5).

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 6 & 18 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 6 & 18.

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

A. Proposed rejection 1: Bischoff anticipates claims 1, 4, 5, 7-11 & 16-17 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.[12] To provide a quick orientation to the Examiner, this section presents an introductory high-level overview of Bischoff's experiments, the steps of the claims, and how Bischoff's experiments map onto each of these steps. A more detailed application of Bischoff's teachings to each claimed step, showing the details of how Bischoff performed each step with specific cites to Bischoff's relevant disclosure is presented in the next section.

[12] 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 '015 patent under 35 U.S.C. § 102(b) (Exhibit PA-1).

Bischoff performed three experiments which can be briefly summarized as followsBischoff 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 made three comparisons:

- **Comparison 1 ("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 at each of two distinct loci (HBB and D11S904).

- **Comparison 2 ("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. For this analysis, Bischoff examined the maternal and paternal 11q CD3D locus.

- **Comparison 3 ("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 at the INFAR locus.

In each of these experiments, Bischoff performed and discoved each of the principal steps of the claims of the ‘015 patent. Generally, the independent method claims (claim 1 and 8) of the '015 patent recite four steps: (1) distributing template molecules from a biological sample to form a set of assay samples (recited in claim 8 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 "*first allelic form of a marker*" and a second number of assay samples that

contain a "*second allelic form of the marker*;" and (4) comparing the two numbers of assay samples to ascertain an allelic imbalance in the biological sample. As an example, Bischoff performed the three main steps of independent claims 8 as follows.

- ***Distributing and/or set-forming step (recited in claim 8 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*" (e.g., claim 8)
  - 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 first allelic form of a marker and a second number of assay samples which contain a second allelic form of the marker.*"
  - Bischoff analyzed the PEP amplification products from each sample to determine whether each parental allele at four different marker loci was present,[13] using four separate secondary locus-specific PCR reactions and gel electrophoresis.

[13] 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).

- 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 first allelic form of a marker.*"

- Bischoff also counted the number of samples containing a second allele of interest, thereby "*determining a second number of assay samples which contain a second allelic form of the marker.*"

- Bischoff chose various different combinations of alleles as the "*first*" and "*second allelic form,*" 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 8 also specifies that the allelic imbalance is "*between the first allelic form and the second allelic form.*"

- 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 between the "*first*" and "*second*" allelic forms of different markers:

  - **Comparison 1 ("intra-locus," two homologous alleles on the suspect "p" arm of chromosome pair 11)** Bischoff compared the number of assay samples containing:

    - a "*first allelic form of a marker*" 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 "*second allelic form of the marker*" 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 2 ("intra-locus," two homologous alleles on the non-suspect "q" arm of chromosome pair 11)**

  - Bischoff compared the number of assay samples containing:

    - a "*first allelic form of a marker*" 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 "*second allelic form of the marker*" in the form of the paternal CD3D allele on paternal chromosome 11.

- **Comparison 3 ("intra-locus," two homologous alleles on non-suspect chromosome pair 21)**

  - Bischoff compared the number of assay samples containing:
    - a "*first allelic form of a marker*" in the form of one maternal allele at the locus INFAR on maternal chromosome 21 and
    - a "*second allelic form of the marker*" 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.[14]

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 claims of the '015 patent are directed to determining the number of assay samples containing a "*first allelic form of a marker*" and a "*second allelic form of the [same] marker*," indicating that "*allelic imbalance*" must encompass imbalances

[14] *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).

between different allelic forms of a single marker.[15] 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.[16]

Bischoff "*determin*[*ed*] *an allelic imbalance*" as recited in the preamble of 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.[17] Bischoff noted that some BWS patients had an allelic imbalance in the form of "partial paternal isodisomy of 11p."[18] 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."[19]

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[15] *See also* U.S. Pat. No. 7,824,889 claim 1, reciting a method for determining an allelic imbalance by determining a first number of assay samples which contain a selected sequence and a second number of assay samples which contain a reference sequence and comparing the two numbers, for which reexamination is concurrently being requested. (Exhibit 7).

[16] *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").

[17] Bischoff, Abstract.

[18] Bischoff, Abstract.

[19] 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.

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 Bischoff's isodisomy (loss of maternal 11p alleles) is relevant to this request** – as explained below, Bischoff determined maternal allelic loss using the claimed methods. [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"), 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.

[20] 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.

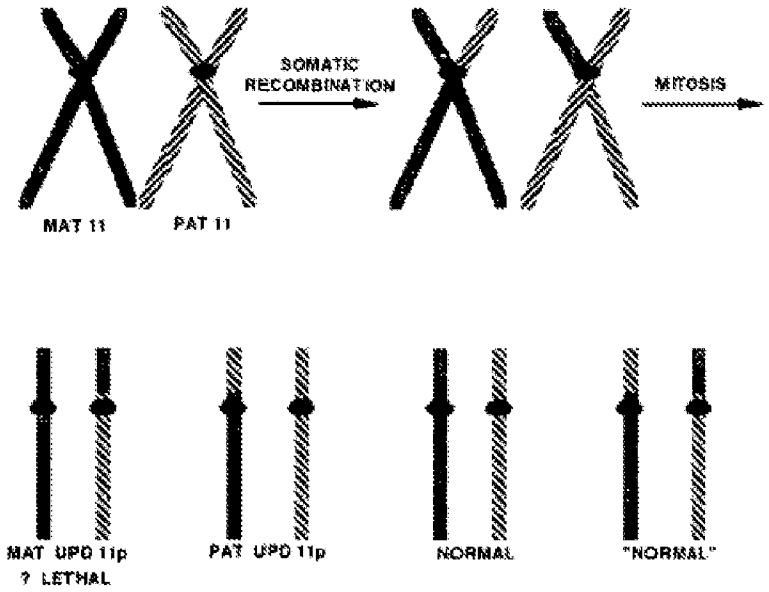

Figure 3. Diagram showing somatic recombination resulting in mosaicism for paternal isodisomy of 11p. Note that the recombination would lead to a population of cells with maternal isodisomy that is presumably lethal and a population of cells that are apparently normal by molecular analysis but contain 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). 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"[21] 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"[22] 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."[23]

Bischoff also determined an allelic imbalance "*in a biological sample.*" In particular, Bischoff analyzed a "blood sample" from his patient,[24] which is explicitly recognized as a preferred biological sample in the '015 patent.[25]

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 the biological sample"*

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

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21 Bischoff, Abstract
22 Bischoff, Abstract
23 Bischoff, Abstract.
24 Bischoff, page 396, right col., last paragraph.
25 '015 patent, col. 7, lines 12-13.

Bischoff started with "*template molecules ... obtained from the biological sample*" as recited in claim 1. First, Bischoff took a "blood sample" from his patient,[26] which is explicitly recognized as a "preferred" biological sample in the '015 patent.[27]

From this biological sample, Bischoff generated *"a set comprising a plurality of assay samples*" containing the "*template molecules" by isolating "single blood lymphocytes*"[28] 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 media and then placed into separate reaction tubes."[29] 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.[30]

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,"[31] in the form of a primer extension preamplification (PEP) reaction of the whole genome."[32] 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

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[26] Bischoff, page 396, right col., last paragraph.
[27] '015 patent, col. 7, lines 12-13.
[28] Bischoff, page 396, right col., last paragraph.
[29] Bischoff, page 398, right col., section titled "Single cell micromanipulation and PEP."
[30] *See, e.g.*, Bischoff, Table 1 (showing analysis of six individual cells)
[31] Bischoff, Abstract.
[32] Bischoff, sentence bridging pages 396-397.

procedure was a form of "[w]hole genome amplification"[33] which "allows for amplification of very small amounts of genetic material"[34] present in single-cell samples. The art recognized PEP as an amplification reaction used to "amplify" genomic DNA, and more specifically recognized PEP as a type of "random PCR."[35] Thus, under the 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,[36] Bischoff successfully detected PEP amplification products of each locus in each of his samples, 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 the biological sample*."

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[33] Bischoff, Abstract.

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

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

[36] Bischoff, Table 2, page 397.

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

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

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

The '015 patent does not expressly define "analyzing the amplified molecules in the assay samples of the set." Instead, the '015 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-labeled probes, and other biochemical assays."[37] Hence, the broadest reasonable interpretation of "*analysis*" encompasses analysis by means of a secondary marker-specific PCR amplification by gel electrophoresis, as performed by Bischoff.

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.[38] The purpose

[37] '015 patent, col. 7, lines 49-56.

[38] 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").

of the locus-specific analysis was "to determine the [parental] chromosome 11 origins"[39] 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.[40] Two 11p "markers HBB and D11S904 were selected for analysis … [as well as two other] informative markers located outside of the BWS region, CD3D on 11q23 and INFAR on chromosome 21."[41] 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.[42]

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.[43]

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

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

[40] 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 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.

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

[42] Bischoff, page 396.

[43] '015 patent, col. 7, lines 49-56. ("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.")

*iv) Bischoff's analysis involved both "**determin[ing]** 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" and "**comparing** the first number to the second number to ascertain an allelic imbalance in the biological sample"*

Bischoff analyzed his samples in several ways, each of which "***determine[d]*** *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*" and "***compar[ed]*** *the first number to the second number to ascertain an allelic imbalance*" as recited in claim 1.

The '015 patent does not provide an explicit definition of "*first allelic form of a marker*" or "*second allelic form of the marker.*" Bischoff's maternal and paternal alleles at a single locus can be regarded as "*first*" and "*second*" allelic forms, or vice versa.

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;[44]

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).

[44] The analysis of both loci are redundant, and Requester will focus mainly on the D11S904 locus going forward. However, Requester's arguments apply equally to the HBB locus.

As discussed below, Bischoff determined a first number and a second number of assay samples containing a first and second allele of interest and comparedthese numbers to 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 first allelic form of a marker and a second number of assay samples which contain a second allelic form of the marker"*

Bischoff determined the presence of all maternal and paternal alleles at his loci as disclosed in Table 2, reproduced below.

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 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.

Thus, under the broadest reasonable interpretation, Table 2 discloses a first number of assay samples containing a first allelic form of a marker and a second number of assay samples containing a second allelic form at each locus that Bischoff analyzed. Each of the single cells taken from the patient child constitutes an assay sample as described previously, above. Each allele can be treated as a "*first*" or a "*second allelic form of a marker*" in downstream comparisons. Treating for example the maternally-inherited D11S904 allele as the "*first allelic form of a marker*" (designated D11S904 allele 4 in Table 2) and the paternally-inherited D11S904 allele (designated D11S904 allele 1 in Table 2) as the "*second allelic form of a marker*," there are 3 assay samples containing the first allelic form and 6 assay samples containing the second allelic form. Alternatively, treating the paternally-inherited D11S904 allele (designated D11S904

allele 1 in Table 2) as the "first allelic form" and the maternally-inherited D11S904 allele (designated D11S904 allele 4 in Table 2) as the second allelic form, there are 6 assay samples containing the first allelic form and 3 assay samples containing the second allelic form.

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.[45] 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."[46] 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.[47]Regardless of which loci (HBB, D11S904, CD3D, and/or INFAR) are chosen, the results in Table 2, along with Bischoff's express statements, disclose the determination of a first number of assay samples containing a first allelic form and a second number of assay samples containing a second allelic form.

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[45] Bischoff, page 398, left col., top paragraph, and Table 2.
[46] Bischoff, page 397, left col., second paragraph , and Table 2.
[47] 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 ("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 first allelic form of a marker*" 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 second allelic form of the marker*" 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)[48]

- 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."[49]

- 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."[50]

For the purposes of **Comparison 2 ("intra-locus," two homologous alleles on the non-suspect "q" arm of chromosome pair 11))** identified in the overview section,

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

Bischoff determined that all six cell samples ("*a first number of assay samples*") contained "*a first allelic form of a marker*" 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 second allelic form*" 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."[51]

- 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."[52]

For the purposes of **Comparison 3 ("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 first allelic form of a marker*" 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 second allelic form*" in the form of the paternally-inherited INFAR allele on paternal chromosome 21 (designated INFAR allele 2 in Table 2), and compared these two numbers:

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

- 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."[53]

- 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."[54]

Therefore, 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 first and second allelic form, 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.[55] Bischoff noted that some BWS patients had an allelic imbalance in the form of "partial paternal isodisomy of 11p"[56] 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

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[53] Bischoff, page 398, left col., top paragraph, and Table 2.
[54] Bischoff, page 397, left col., second paragraph , and Table 2.
[55] Bischoff, Abstract.
[56] Bischoff, Abstract.

region. In addition, the isodisomy in Bischoff's particular patient also "involv [ed] loss of the maternal 11p region in some cells."[57]

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 Bischoff's isodisomy (loss of maternal 11p alleles) is relevant to this request** – as explained below, Bischoff checked for maternal allelic loss using the claimed methods.[58]

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"[59] in which some cells in the patient sample were isodisomic and had lost the maternal allele, but other cells in the sample were genetically normal with "normal biparental inheritance"[60] 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

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[57] 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.

[58] 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 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.

[59] Bischoff, Abstract.

[60] Bischoff, Abstract.

from each parent.[61] 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."[62]

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 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

[61] Bischoff, page 397, Table 2.
[62] Bischoff, Abstract.

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.*"[63] The second amplification reaction was a locus-specific PCR reaction in which the PEP-amplified molecules were analyzed. Thus, under the broadest reasonable interpretation, Bischoff's secondary locus-specific amplification generates the "*amplification product*" mentioned in the analyzing step, that 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 or loci. Dependent claim 4, which is necessarily included within the scope of base claim 1, states that "*between 0.1 and 0.9 of the assay samples yield an amplification product* "as determined by amplification of the first allelic form. Solely for the purposes of this reexamination, Requester will proceed on the premise that by amplifying a "*first allelic form of a marker*" in 0.5 (*i.e.*, between 0.1 and 0.9) of his assay

[63] *See* Section (VI)(A)(2)(ii) above.

samples as explained below, Bischoff necessarily anticipates dependent claim 4 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 both the maternal and paternal alleles "in cells 2, 3 and 4 with the [same] 11p markers."[64] Taking the maternal D11S904 allele as the "*first allelic form of a marker*," 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 first allelic form. Taking the maternal allele at the 11p locus HBB as the "*first allelic form*" yields the same result: three of six (*i.e.*, 0.5) assay samples were found to contain the first allelic form. 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.

vi) *Bischoff succeeded in "identifying an allelic imbalance in the biological sample"*

By performing the claimed steps, Bischoff "*identif[ied]an allelic imbalance in the biological sample*" as recited in claim 1. Bischoff determined the presence of an allelic imbalance as a loss of maternal 11p region in his biological sample. In particular,

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

Bischoff 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."[65] Bischoff referred to this result as "partial paternal isodisomy of 11p."[66]

As explained, 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 – in particular, Bischoff determined maternal allelic loss using the claimed methods.[67]

Because paternal 11p isodisomy involves loss of maternal alleles that were originally present on the "p" arm of chromosome 11, Bischoff's partial paternal isodisomy is an "allelic imbalance." Accordingly, Bischoff successfully "*identif[ied] an allelic imbalance in the biological sample*" as recited in claim 1.

3. Detailed explanation of the pertinency and manner of applying Bischoff to independent claim 8

Independent claim 8 is substantially identical to independent claim 1, with the following main differences:

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[65] Bischoff, page 397, left col., second paragraph, and Table 2.

[66] Bischoff, Abstract.

[67] 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.

- Claim 8 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 8 does not require that amplification takes place in "each" assay sample;
- In contrast to claim 1, claim 8 does not require that "between 0.1 and 0.9 of the assay samples yield an amplification product" of a first or second allelic form;
- Claim 8 explicitly specifies that the allelic imbalance is between the first allelic form and the second allelic form; and
- Claim 8 does not require that an allelic imbalance is actually identified in the biological sample.

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 8.

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."[68] 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."[69]

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. Thus, 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 8.

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 8.

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

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

v) *Bischoff's analysis involved both "**determin[ing]** 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" and "**comparing** the first number ... 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."*

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 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 first number ... to the second number of assay samples to ascertain an allelic imbalance ... in the biological sample*," as also required by claim 8. Bischoff also anticipates the additional language found in this limitation in claim 8.

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

In contrast to claim 1, claim 8 additionally specifies that the allelic imbalance is "*between the first allelic form and the second allelic form,*" which was not explicitly stated in claim 1.

Bischoff ascertained an allelic imbalance "*between the first allelic form and the second allelic form*" as recited in claim 8. For example in **Comparison 1 ("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 (allele 4), situated on the suspect "p" arm of maternal chromosome 11 (*i.e.*, "*a first allelic form*"). In contrast, all six samples (a "*second number")* contained the paternally-inherited

D11S904 allele (allele 1) on paternal chromosome 11 (*i.e.*, "*a second allelic form*"), and compared these two numbers.[70]

By ascertaining that an allelic imbalance existed between two alleles at the D11S904 locus, one situated on the maternal chromosome and the other allele situated on the paternal chromosome, Bischoff thereby ascertained that an allelic imbalance existed between the first and second allelic forms.

Bischoff therefore ascertained an allelic imbalance "*between the first allelic form and the second allelic form,*" as recited in claim 8.

4. Detailed explanation of the pertinency and manner of applying Bischoff to claims 7 and 9

Dependent claim 7 recites the method of claim 1 "*wherein the sample is from blood.*" Dependent claim 9 recites the method of claim 8 "*wherein the sample is from blood.*"

As explained above, Bischoff anticipates base claims 1 and 8. 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 …".[71]

Accordingly, Bischoff anticipates claims 7 and 9 as well as base claims 1 and 8.

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

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

5. Detailed explanation of the pertinency and manner of applying Bischoff to claims 4, 5, 10, 11, 16 and 17

Dependent claims 4, 5, 10, 11, 16 and 17 all recite the method of claim 1 and/or claim 8 wherein a specified subpopulation of the assay samples "*yield an amplification product*." Under the broadest reasonable interpretation, Bischoff anticipates these claims.

i) *Anticipation of claims 10 and 11*

Dependent claims 10 and 11 recite the method of claims 1 or 8, where "*between 0.1 and 0.6*" (claim 10) or "*between 0.3 and 0.5*" (claim 11) "*of the assay samples yield an amplification product*," without specifying what particular template sequence the amplification product is generated from.

Base claim 1 makes clear that "an amplification product" as recited in claims 10 and 11 is separate and distinct from the "population of amplified molecules" generated in the amplifying step of the base claims. As discussed 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 of claim 1 must necessarily be separate and distinct from the "*population of amplified molecules*" generated in the amplifying step of claim 1, 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.

Under the broadest reasonable interpretation, "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.

Although claims 10 and 11 fail to specify that the "amplification product" has any particular sequence, dependent claim 16, which also depends from claim 1, has identical language to claims 10 and 11 and further specifies that a certain portion of the assay samples yield an amplification product "*as determined by amplification of the first allelic form of the marker*." Thus, claims 10 and 11 should be anticipated if "*between 0.1 and 0.6*" (claim 10) or "*between 0.3 and 0.5*" (claim 11) "*of the assay samples yield an amplification product*" as determined by amplification of the first allelic form of the marker.

Analyzing the products of his secondary locus-specific amplification reaction, Bischoff found that three of six single-cell assay samples yielded an amplification product as determined by amplification of the first allelic form of a marker (specifically, the maternal HBB allele). Thus, 0.5 (*i.e.*, three of six) assay samples yielded "an amplification product" as determined by the maternal HBB allele. Bischoff also found the same results when examining the D11S904 locus.

Because claims 10 and 11 allow "*an amplification product*" to be amplified from any particular sequence including Bischoff's maternal HBB allele or maternal D11S904 allele, and because Bischoff saw maternal amplification product of the HBB or D11S904 marker in 0.5 (i.e., between 0.1 and 0.9) of his assay samples, Bischoff anticipates claim 10 and 11 in addition to base claim 8.

ii) *Anticipation of claims 4, 5, 16 and 17*

Dependent claims 4 and 16 specify that "*between 0.1 and 0.9 of the assay samples yield an amplification product as determined by amplification of the first allelic form of a marker*." Dependent claims 5 and 17 specify that "*between 0.1 and 0.9 of the assay samples yield an amplification product as determined by amplification of the second allelic form*." Claims 4 and 5 depend on claim 1 and claims 16 and 17 depend on claim 8, and both base claims are anticipated by Bischoff as discussed above.

Bischoff anticipates claims 4 and 16. Bischoff analyzed his PEP amplification products by a secondary analytical locus-specific PCR reaction, and more specifically checked for "*an amplification product as determined by amplification of the first allelic form of a marker*" in the form of the maternal D11S904 allele (D11S904 allele 4 in Table 2) and the maternal HBB allele (HBB allele 2) both on chromosome 11, which he suspected was lost in a subset of cells. Bischoff found that only three of six single-cell assay samples yielded an "*amplification product as determined by amplification of the first allelic form of a marker*" in the form of the maternal D11S904 and HBB alleles. In particular, Bischoff found in "cells numbered 1, 5 and 6 … only the paternal allele" showed a (secondary) amplification product at the D11S904 and HBB loci whereas "[n]ormal biparental inheritance" - *i.e.*, presence of both the maternal and paternal alleles - was detected "in cells 2, 3 and 4."[72] The paternal D11S904 and HBB alleles would be the second allelic form of the marker here, respectively. Thus, three of six or 0.5 assay samples yielded "*an amplification product as determined by amplification of the first allelic form of a marker*" of the maternal D11S904 or HBB alleles. Because 0.5 is

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

"*between 0.1 and 0.9 of the assay samples*," and because Bischoff anticipates both claims 1 and 8 as described above, Bischoff also thereby anticipates claims 4 and 16.

Similarly, dependent claims 5 and 17 specify that "*between 0.1 and 0.9 of the assay samples yield an amplification product as determined by amplification of the second allelic form*." Bischoff made various comparisons between various sequences and his maternal D11S904 and HBB alleles, thereby treating the maternal D11S904 allele (D11S904 allele 4 in Table 2) or the maternal HBB allele (HBB allele 2 in Table 2) as a "*second allelic form*" under the broadest reasonable interpretation. Because Bischoff saw maternal D11S904 or maternal HBB amplification product in 0.5 of his assay samples, Bischoff anticipates these claims as well.

Accordingly, Bischoff anticipates claims 4, 5, 10, 11, 16 and 17 in addition to anticipating their base claims 1 and 8.

B. Proposed rejection 2: Bischoff renders obvious claims 2, 3, 14, and 15 in view of Kalinina under 35 U.S.C. § 103(a)

Dependent claims 2 and 14 recites the method of base claims 1 and 8, wherein "*the step of amplifying employs real-time polymerase chain reactions*." Dependent claims 3 and 15 recite the method of base claims 1 and 8 "*wherein the real-time polymerase chain reactions comprise a dual-labeled fluorogenic probe*." All claims are obvious over Bischoff in view of Kalinina.[73]

As discussed above, Bischoff anticipates base claims 1 and 8, by isolating single cells and performing a PEP amplification step. This amplification increases the amount

[73] Kalinina *et al.*, *Nanoliter scale PCR with TaqMan detection*. Nucleic Acids Res. 25(10):1999-2004 (May 15, 1997), forming prior art to the '015 patent under 35 U.S.C. § 102(b) (Exhibit PA-2).

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 14. 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.[74] Both of these moieties are fluorogenic.[75] The '015 patent explicitly recognizes that TaqMan® probes are "dual-labeled fluorogenic probes,"[76] and are used in real-time PCR reactions.[77]

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.[78] As amplification progresses, the dual-labeled probe will hybridize to the amplified target sequence and the reporter molecule will be cleaved from the probe by Taq polymerase, resulting in an increase in fluorescence of the reporter.[79]

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

[75] *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.")

[76] '015 patent, col. 7, lines 49-56.

[77] '015 patent, col. 5, lines 34-39.

[78] Kalinina, Abstract.

[79] Kalinina, page 1999, left col. (Introduction).

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,"[80] such as Bischoff's amplification products.

It would have been obvious to combine use of the TaqMan® assay described in Kalinina in single-cell amplification and determination of allelic imbalance as taught by 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 (*e.g.*, Bischoff's methods were intended to detect the presence of a single template sequence molecule in the form of a paternal 11p allele).[81] 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 starting from one (or two) template molecule(s).[82]

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

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[80] Kalinina,page 2003.

[81] Kalinina, Abstract.

[82] Kalinina, page 2001, right col. ("Human DNA was diluted so that PCRs contained 0-14 haploid genome equivalents (0-42 pg)/capillary.")

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*." 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 '015 patent. Kalinina's data also indicated that her 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.[83] For example, Table 1 in Kalinina shows that for a small capillary diameter (25 or 30 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 (which corresponds to 1.5 templates) is slightly less than that of Bischoff, whose

[83] Kalinina, Table 1 (reactions with at least 1.5 haploid genomes equivalens/capillary in smaller capillaries (20 and 30 μm diameters).). 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 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").

assay samples each contained 1 diploid genome (*i.e.*, the equivalent of 2 haploid genomes, containing two template molecules).

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 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.[84] 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 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). (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 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).

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[84] 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.")

Thus, a person using Kalinina's TaqMan®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 '015 claims therefore embody a merely predictable substitution of Kalinina's TaqMan® procedure for Bischoff's PEP amplification procedure for single-cell PCR as taught by Bischoff's.

Therefore, it would have been obvious to the skilled person to have used the TaqMan® assays to analyze single cells as taught by 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. The '015 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 single-cell amplification format to obtain predictable results.[85] While Bischoff used a more complicated procedure than Kalinina's TaqMan® procedure in order to analyze multiple loci on a single cell (a PEP amplification followed by locus-specific PCR reaction and overnight autoradiography), it would have been obvious to amplify different single loci using TaqMan® probes to arrive at the same results. Thus, claims 2, 3, 14, and

[85] '015 patent, col. 7, lines 49-56 ("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.),").

15 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, 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."[86] 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® apparatus described in Kalinina to perform the amplification and detection/analysis of DNA sequences in cells with predictable results.

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[86] 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.[87] 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."[88] Bischoff used a more complicated 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, 14, and 15 obvious.

C. Proposed rejection 3: Bischoff renders claims 12 & 13 obvious in view of Li under 35 U.S.C. § 103(a)

Dependent claims 12 and 13 recite the method of claims 1 or 8, "*wherein the set comprises at least*" 500 (claim 12) or 1000 (claim 13) "*assay samples*."

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[87] *See* MPEP at §2143(C).
[88] Kalinina, page 1999.

Under the broadest reasonable interpretation, Bischoff renders claims 12-13 obvious in view of Li.[89] Bischoff anticipates base claims 1 and 8 as explained previously. Li teaches or suggests single-cell PCR on greater than 500 or greater than 1000 assay samples, as recited in claims 12-13 respectively.

Specifically, Li teaches the method of single-cell PCR on both haploid (sperm) and diploid cells and made and analyzed a set of single-cell assay samples by single-cell PCR, and checked for imbalance in allelic representations in the form of segregation distortion in haploid cells.[90] 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 12-13

In prosecution of a pending continuation of the '015 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,***[91] 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

[89] 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 '015 patent under 35 U.S.C. § 102(b) (Exhibit PA-3).

[90] 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.

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

> "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, because recombination happens at a rate of 1% per million base pairs.[92] 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."[93] 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

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[92] 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 …").

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

Bischoff. Thus, dependent claims 12 and 13 are *prima facie* obvious over Bischoff in view of Li.

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 a large number of samples as suggested by Li. 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. The '015 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

using single-cell PCR. 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."[94] 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. When analyzing loci for such mild allelic imbalance, it would thus have been obvious to one of ordinary skill to use a large number of assay samples in Bischoff's single-cell analysis to ensure that mild forms of allelic imbalance would be detected.

Thus, dependent claims 12 and 13 are *prima facie* obvious over Bischoff in view of Li.

D. Proposed rejection 4: Bischoff renders claims 6 & 18 obvious in view of Ruano II under 35 U.S.C. § 103(a)

Dependent claims 6 and 18 recites the methods of base claims 1 and 8, respectively, "*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 second allelic form of the marker and the second number of assay samples do not contain the first allelic form of the marker*." Bischoff

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

anticipates base claims 1 and 8, as discussed above. In addition, Bischoff renders claims 6 and 18 obvious in view of Ruano II[95] under the broadest reasonable interpretation.

In particular, Bischoff anticipates base claims 1 and 8 by teaching the use of single-cell PCR to distinguish between two 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 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*" that meets the requirements of claims 6 & 18. Ruano II teaches allele-specific PCR as an alternative amplification method that differentiates between two 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

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[95] Ruano *et al.*, Nucleic Acids Res. 17(20):8392 (Oct 25, 1989), which forms prior art to the '015 patent under 35 U.S.C. § 102(b) (Exhibit PA-4).

site."[96] 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 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 (+)."[97] 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").[98] 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)."[99] 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

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[96] Ruano II, page 8392, first paragraph.
[97] Ruano II, page 8392, third paragraph, and Fig. 1(b).
[98] Ruano II, page 8392, third paragraph, and Fig. 1(b).
[99] Ruano II, page 8392, second paragraph.

3,+."[100] 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 repeats.[101] Bischoff similarly analyzed polymorphic loci that were also "dinucleotide repeat markers."[102]

Whereas Bischoff anticipates base claims 1 and 8, Ruano II's allele-specific amplification meets the added limitations of dependent claims 6 and 18. In particular, Ruano II's allele-specific amplification yields amplified molecules of only one allele and not the other, such that 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 second allelic form of the marker and the second number of assay samples do not contain the first allelic form of the marker.*"

It would have been obvious to assess allelic imbalance using Bischoff's single-cell amplification strategy using allele specific primers as taught by Ruano II, where such primers would amplify only a single allele, thereby producing amplified molecules that are "*homogenous*" as required by claims 6 and 18, 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*

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[100] Ruano II, page 8392, second paragraph.

[101] 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).

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

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.

It would have been obvious to use Ruano'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. Ruano'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.[103] Although Bischoff followed up by analyzing multiple different loci both inside and outside the imbalanced 11p portion of

[103] 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.")

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 Ruano's 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.

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 '015 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 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,[104] 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.

[104] Bischoff, Figs. 1 and 2.

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 6 and 18 are *prima facie* obvious over Bischoff in view of Ruano II.

VII. CONCLUSION

Claims 1-18 of the '015 patent are anticipated under 35 U.S.C. § 102(b) over Bischoff, or alternatively rendered obvious over Bischoff. Accordingly, reexamination of claims 1-18 of the '015 patent is respectfully requested.

VIII. CONCURRENT LITIGATION AND REEXAMINATION PROCEEDINGS

The '015 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)).

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 3.

Dated: June 17, 2013

Respectfully submitted,

By: /Ashita A. Doshi/

Reg. No. 57,327

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5791 Van Allen Way
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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 3

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:	16043476
Application Number:	90012896
International Application Number:	
Confirmation Number:	8361
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 3
Receipt Date:	17-JUN-2013
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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	4186
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	LT00831REX3-Exhibit1-US7915015.pdf	1348418 58a8e2f33cff98c7652d2b8628c69beff8de3098	no	21
Warnings:					
Information:					
2	Reexam - Affidavit/Decl/Exhibit Filed by 3rd Party	LT00831REX3-Exhibit3-US7915015-file-history.pdf	5797641 7c445dd4eaac7e1a3ca2364ddbdf048edbc7411b	no	164
Warnings:					
Information:					
3	Reexam - Affidavit/Decl/Exhibit Filed by 3rd Party	LT00831REX3-Exhibit4-US6440706-file-history.pdf	9074494 09e3de4b8668f87a72dc438d04368e979ab64c3c	no	207
Warnings:					
Information:					
4	Reexam - Affidavit/Decl/Exhibit Filed by 3rd Party	LT00831REX3-Exhibit5-Lapidus-US5928870.pdf	1186457 d45c30ef11e95e6354e2c65f4c12450896493640	no	15
Warnings:					
Information:					
5	Reexam - Affidavit/Decl/Exhibit Filed by 3rd Party	LT00831REX3-Exhibit6-Ruano-1990.pdf	9468550 e84374bd97a0146ddc0f1f26c93b8109f2d03ce7	no	6
Warnings:					
Information:					
6	Reexam - Affidavit/Decl/Exhibit Filed by 3rd Party	LT00831REX3-Exhibit7-US7824889.pdf	22665779 4785edee30388e83cf25c65293ef1741fd2e3d33	no	20
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7	Reexam - Affidavit/Decl/Exhibit Filed by 3rd Party	LT00831REX3-Exhibit8-Brenner-1995.pdf	8180319 b725e7d588990fba3c6339f50012370bb7abb762	no	5
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Information:					
8	Reexam - Affidavit/Decl/Exhibit Filed by 3rd Party	LT00831REX3-Exhibit9-Cheung-1996.pdf	88801 066386b5bff552663b5c7f561a8cde4a3679993b	no	5
Warnings:					

Information:					
9	Reexam - Affidavit/Decl/Exhibit Filed by 3rd Party	LT00831REX3-Exhibit10-vonEggeling-1997.pdf	1288326 c0b4ae3b5599f76e2a608bc03b8b29edb468976f	no	6
Warnings:					
Information:					
10	Reexam - Affidavit/Decl/Exhibit Filed by 3rd Party	LT00831REX3-Exhibit11-13071105-file-history.pdf	12533202 78119a855fb5f054bff8795979818a3af7995e10	no	189
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11	Non Patent Literature	LT00831REX3-ExhibitPA-1-Bischoff-1995.pdf	10290997 62cbcb68a7172155975fc7edbc944b56534df175	no	6
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Information:					
12	Non Patent Literature	LT00831REX3-ExhibitPA-2-Kalinina-1997.pdf	9160479 fa7ec83d92e934cc405d1531f1fd6e7b97c1df1a	no	7
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Information:					
13	Reexam - Affidavit/Decl/Exhibit Filed by 3rd Party	LT00831REX3-ExhibitPA-3-Li-1988.pdf	471489 60830cac74e4da8e2dc46a3a742327c954093503	no	5
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Warnings:					
Information:					
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		Total Files Size (in bytes):	93562689		

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New International Application Filed with the USPTO as a Receiving Office

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EXHIBIT 1

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.

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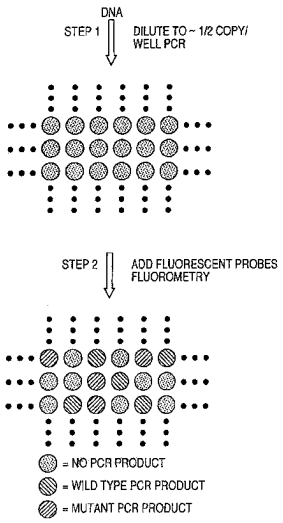
Primary Examiner — Samuel C Woolwine
(74) *Attorney, Agent, or Firm* — Banner & Witcoff, Ltd.

(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.

18 Claims, 7 Drawing Sheets



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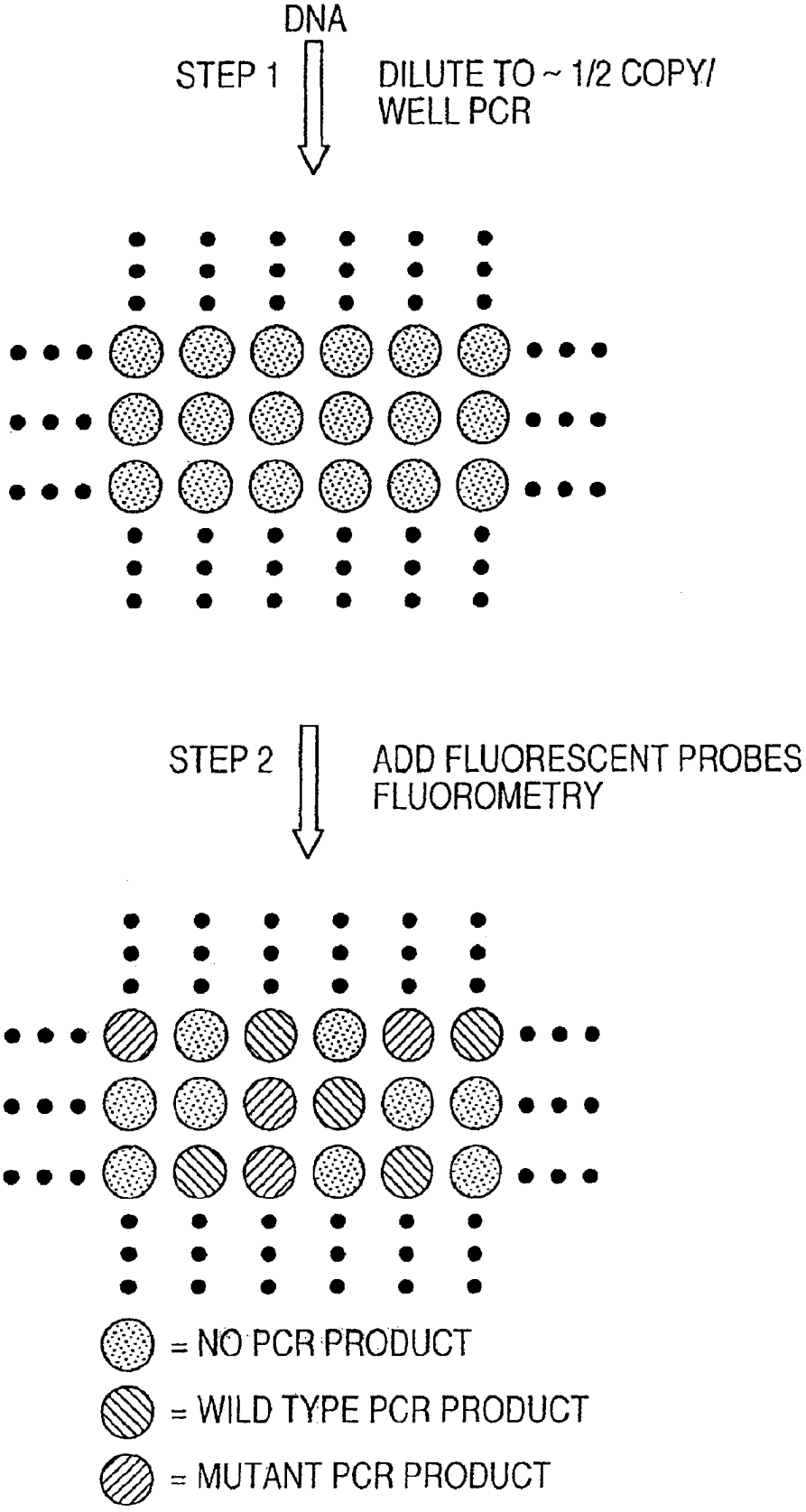
Office Action dated Jun. 5, 2009 in co-pending U.S. Appl. No. 11/709,742.

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

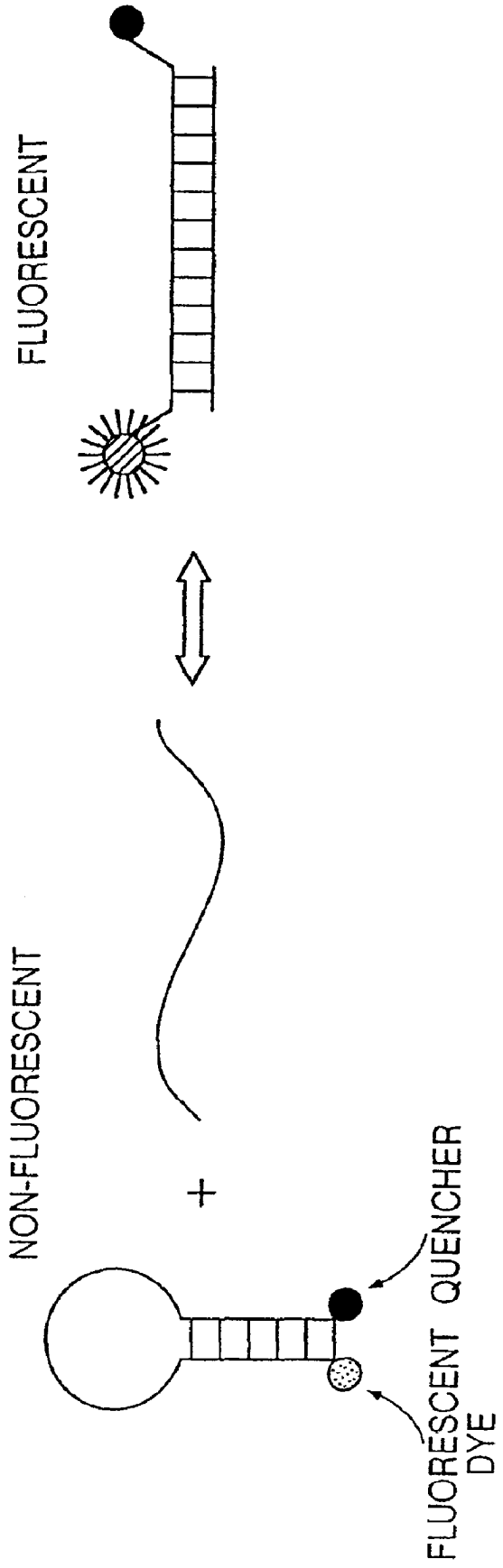


FIG. 1B

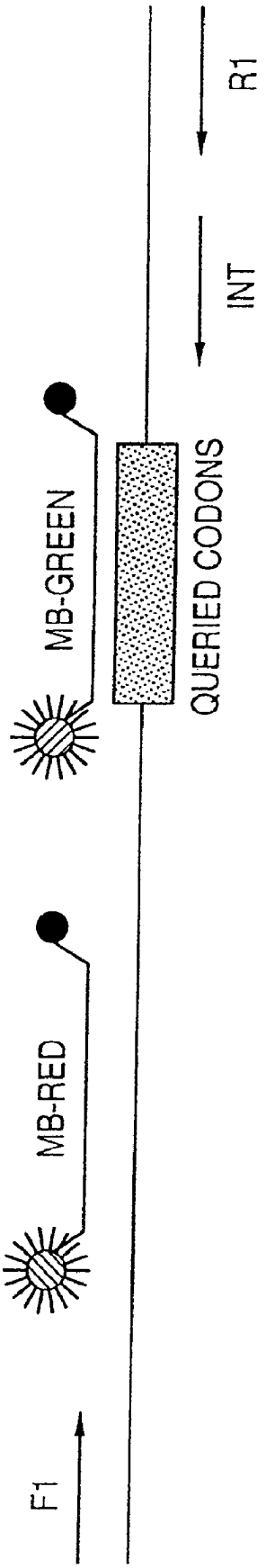


FIG. 1C

FIG. 2

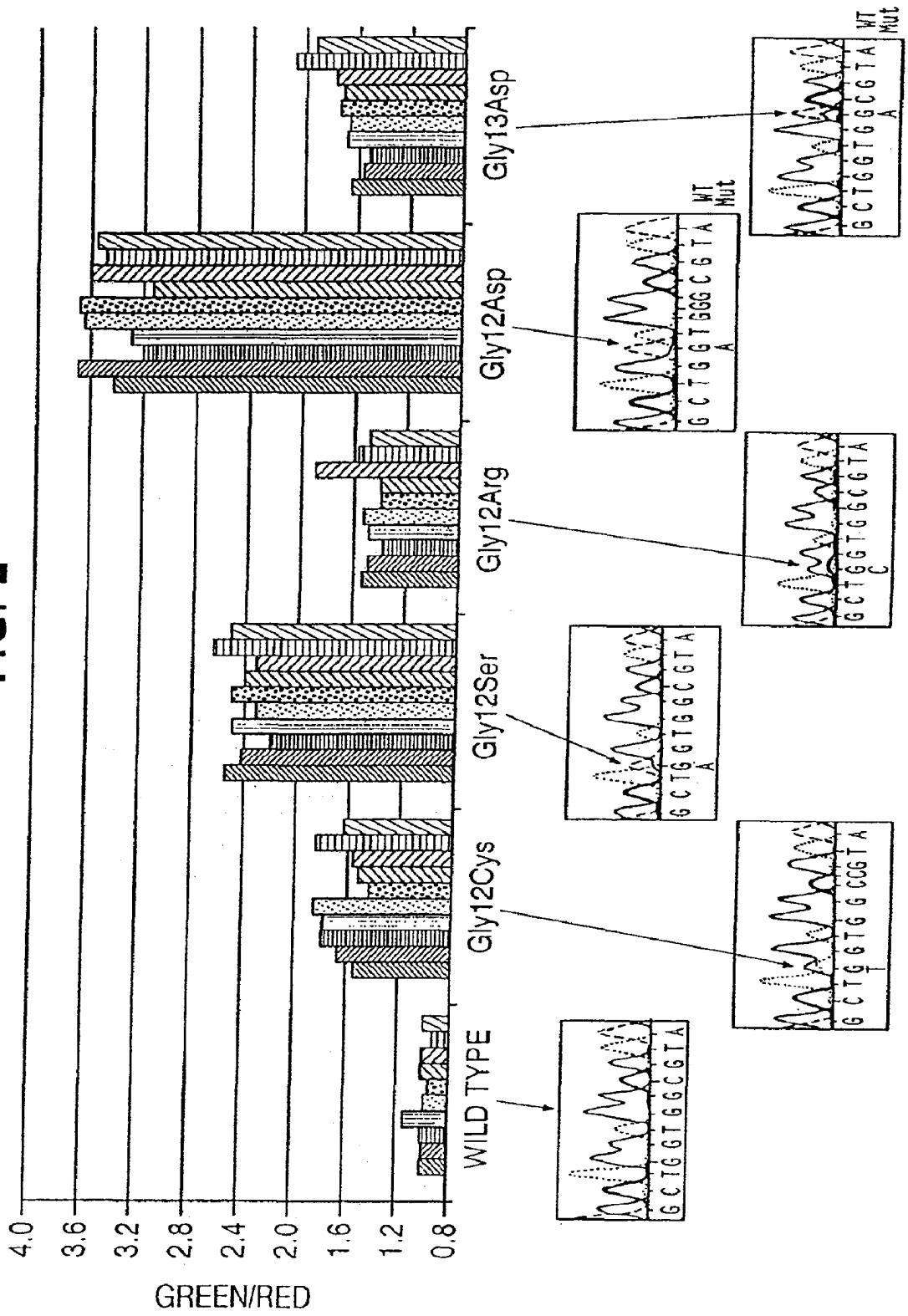
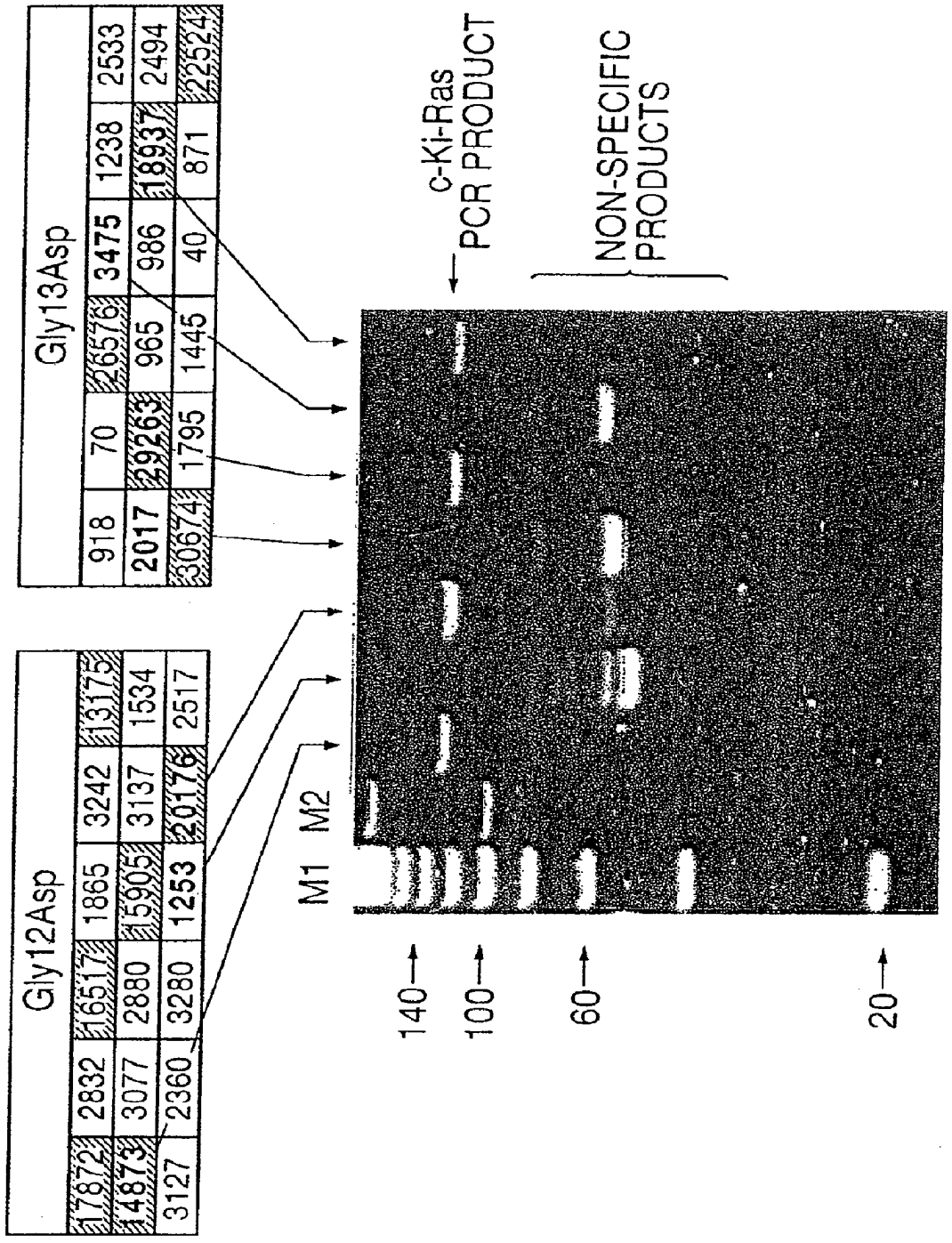

Gly13Asp
Gly12Asp
Gly12Arg
Gly12Ser
Gly12Cys
WILD TYPE
4.0
3.6
3.2
2.8
2.4
2.0
1.6
1.2
0.8
GREEN/RED
WT
Mut

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

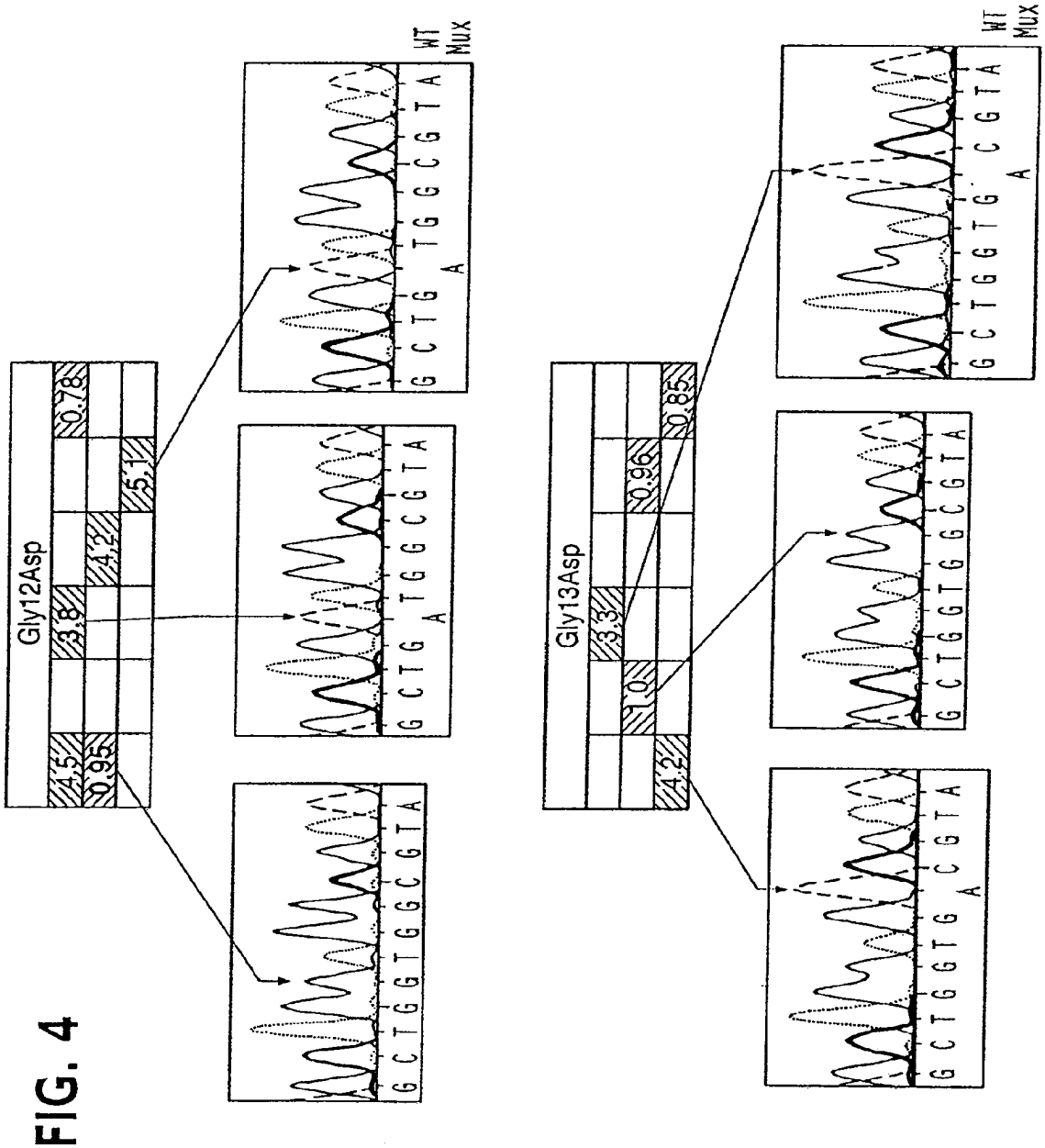
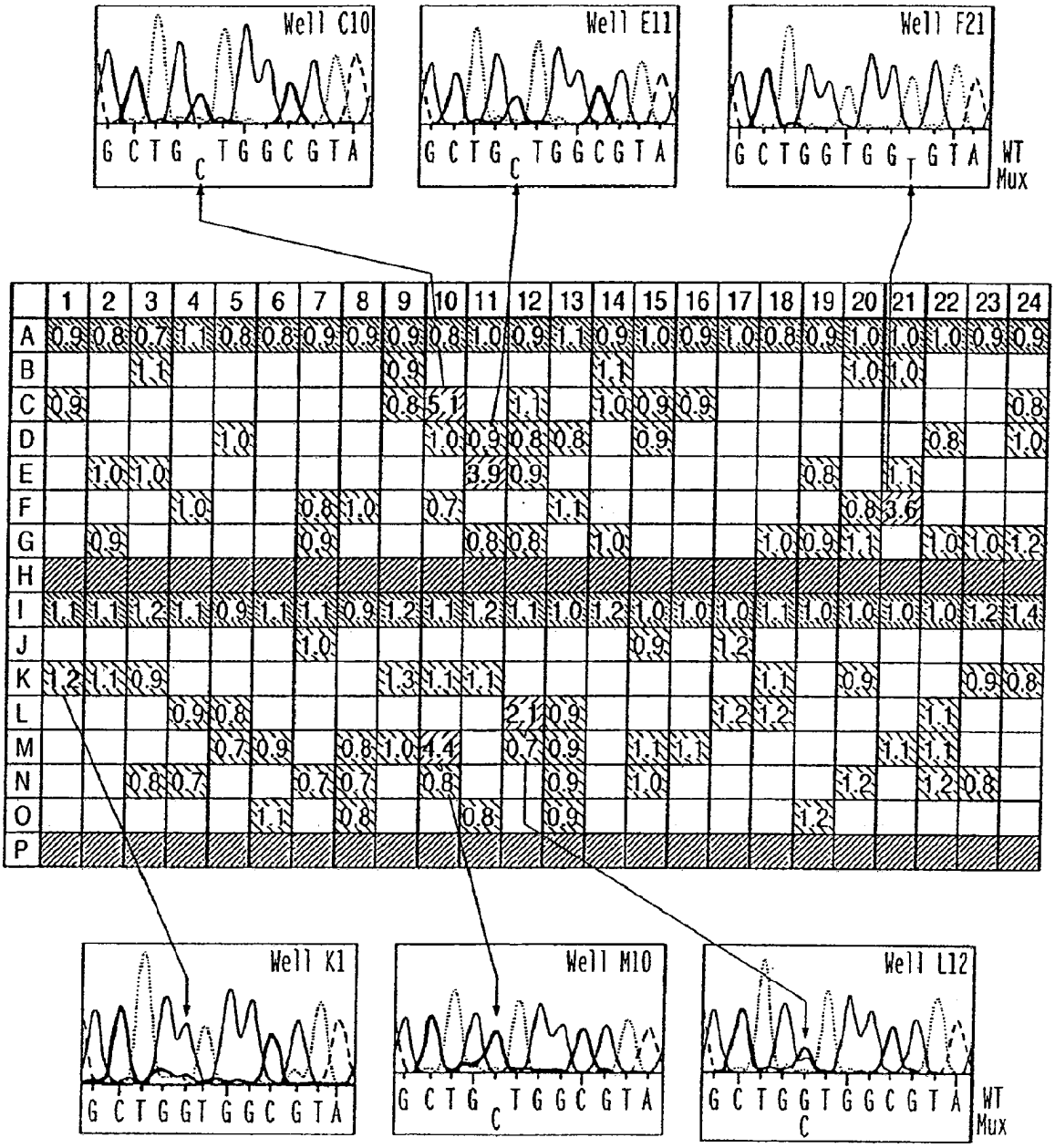

Gly12Asp
0.78
5.1
4.2
3.8
4.5
0.95
G C T G G T G G C G T A
G C T G T G G C G T A
A
G C T G T G C G T A
A
WT
MUX
Gly13Asp
0.85
0.96
3.3
1.0
4.2
G C T G G T G G C G T A
A
G C T G G T G G C G T A
G C T G G T G C G T A
A
WT
MUX

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 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% ($\frac{4}{102}$) 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 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.

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Filed: November 12, 2009

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
06 October 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.

EXHIBIT 3

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	PATENT NUMBER	GROUP ART UNIT	FILE WRAPPER LOCATION
12/617,368	7915015	1637	9200

OC000000050585408

Correspondence Address/Fee Address Change

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

- **Correspondence Address**

The address of record for Customer Number 11332 is:

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

PART 1 - ATTORNEY/APPLICANT COPY
page 1 of 1

UNITED STATES PATENT AND TRADEMARK OFFICE

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

APPLICATION NO.	ISSUE DATE	PATENT NO.	ATTORNEY DOCKET NO.	CONFIRMATION NO.
12/617,368	03/29/2011	7915015	001107.00794	4461

22907 7590 03/09/2011

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

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

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

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

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

BERT VOGELSTEIN, BALTIMORE, MD;
KENNETH W. KINZLER, BALTIMORE, MD;

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
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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 12/16/2010

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.
12/617,368	11/12/2009	BERT VOGELSTEIN	001107.00794	4461

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	NO	$1510	$300	$0	$1810	03/16/2011

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:
☒ 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 19-0733 (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 February 16, 2011

Typed or printed name Sarah A. Kagan Registration No. 32,141

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.

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PTOL-85 (Rev. 08/07) Approved for use through 08/31/2010. OMB 0651-0033 U.S. Patent and Trademark Office; U.S. DEPARTMENT OF COMMERCE

Electronic Patent Application Fee Transmittal

Application Number:	12617368
Filing Date:	12-Nov-2009
Title of Invention:	DIGITAL AMPLIFICATION
First Named Inventor/Applicant Name:	BERT VOGELSTEIN
Filer:	Sarah Anne Kagan./Daphne Cashion
Attorney Docket Number:	001107.00794

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:	9458465
Application Number:	12617368
International Application Number:	
Confirmation Number:	4461
Title of Invention:	DIGITAL AMPLIFICATION
First Named Inventor/Applicant Name:	BERT VOGELSTEIN
Customer Number:	22907
Filer:	Sarah Anne Kagan./Daphne Cashion
Filer Authorized By:	Sarah Anne Kagan.
Attorney Docket Number:	001107.00794
Receipt Date:	16-FEB-2011
Filing Date:	12-NOV-2009
Time Stamp:	14:58:12
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	1265
Deposit Account	190733
Authorized User	

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

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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)	001107_00794_Issue_Fee_Transmittal_02_16_2011.pdf	108775 9372983c2cee2360a50f6ed01cf3dca04dc15128	no	1
Warnings:					
Information:					
2	Fee Worksheet (PTO-875)	fee-info.pdf	32018 8c0d60c6eead5ec4ac1bcab9f478c7d1f5c5f698	no	2
Warnings:					
Information:					
		Total Files Size (in bytes):	140793		

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

New Applications Under 35 U.S.C. 111

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

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

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

New International Application Filed with the USPTO as a Receiving Office

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

~~IN THE SPECIFICATION~~

Please replace the paragraph beginning on page ~~4, line 16~~: 22 MM

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.

8 MM IDC-a1,AMD

~~Please replace the paragraph beginning page 5, line 5.~~

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

IDC-a2,AMD,M

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NOTICE OF ALLOWANCE AND FEE(S) DUE

22907 7590 12/16/2010

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: 12/16/2010

APPLICATION NO.	FILING DATE	FIRST NAMED INVENTOR	ATTORNEY DOCKET NO.	CONFIRMATION NO.
12/617,368	11/12/2009	BERT VOGELSTEIN	001107.00794	4461

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	NO	$1510	$300	$0	$1810	03/16/2011

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.

PTOL-85 (Rev. 08/07) Approved for use through 08/31/2010.

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
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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 12/16/2010

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

Certificate of Mailing or Transmission

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

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

APPLICATION NO.	FILING DATE	FIRST NAMED INVENTOR	ATTORNEY DOCKET NO.	CONFIRMATION NO.
12/617,368	11/12/2009	BERT VOGELSTEIN	001107.00794	4461

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	NO	$1510	$300	$0	$1810	03/16/2011

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.

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APPLICATION NO.	FILING DATE	FIRST NAMED INVENTOR	ATTORNEY DOCKET NO.	CONFIRMATION NO.
12/617,368	11/12/2009	BERT VOGELSTEIN	001107.00794	4461

22907 7590 12/16/2010

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: 12/16/2010

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 0 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 0 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)	
	12/617,368	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 *papers filed 10/06/2010 and 10/13/2010*.

2. ☒ The allowed claim(s) is/are *1-18*.

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 _____
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

Issue Classification	Application/Control No.	Applicant(s)/Patent Under Reexamination
12617368	12617368	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	24.3	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	1	17	17												
2	2	18	18												
3	3														
4	4														
5	5														
6	6														
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12	12														
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14	14														
15	15														
16	16														

NONE		Total Claims Allowed:	
(Assistant Examiner)	(Date)	18	
/SAMUEL C WOOLWINE/ Primary Examiner.Art Unit 1637	12/05/2010	O.G. Print Claim(s)	O.G. Print Figure
(Primary Examiner)	(Date)	1	1A

Search Notes	Application/Control No.	Applicant(s)/Patent Under Reexamination
12617368	12617368	VOGELSTEIN ET AL.
	Examiner	**Art Unit**
	SAMUEL C WOOLWINE	1637

SEARCHED			
Class	**Subclass**	**Date**	**Examiner**

SEARCH NOTES		
Search Notes	**Date**	**Examiner**
Inventor name, keyword search in EAST, Google Scholar (see printouts); review prosecution history of parent applications; consideration of IDSs.	09/21/2010	SCW

INTERFERENCE SEARCH			
Class	**Subclass**	**Date**	**Examiner**
	Keyword search in EAST	12/05/2010	SCW

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

BIB DATA SHEET

CONFIRMATION NO. 4461

SERIAL NUMBER	FILING or 371(c) DATE	CLASS	GROUP ART UNIT	ATTORNEY DOCKET NO.
12/617,368	11/12/2009 RULE	435	1637	001107.00794

APPLICANTS

BERT VOGELSTEIN, BALTIMORE, MD;
KENNETH W. KINZLER, BALTIMORE, MD;

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

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

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

**** IF REQUIRED, FOREIGN FILING LICENSE GRANTED ****

05/06/2010

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	13	2

ADDRESS

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

TITLE

Digital Amplification

FILING FEE RECEIVED		
1584	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)

<This search history is empty>

EAST Search History (Interference)

Ref #	Hits	Search Query	DBs	Default Operator	Plurals	Time Stamp
L1	1	allelic.clm. and imbalance.clm. and amplif$7. clm. and compar$3.clm. and (samples aliquots portions).clm. and number. clm.	USPAT; UPAD	OR	OFF	2010/12/05 15:23

12/5/2010 3:23:45 PM

Application Number	Application/Control No.	Applicant(s)/Patent under Reexamination	
	12/617,368	VOGELSTEIN ET AL.	

Document Code - DISQ	Internal Document – DO NOT MAIL

TERMINAL DISCLAIMER	☒ APPROVED	☐ DISAPPROVED
Date Filed : 10/06/10	**This patent is subject to a Terminal Disclaimer**	

Approved/Disapproved by:

Felicia D. Roberts

6,440,706

U.S. Patent and Trademark Office

PATENT

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re Application of	)	Prior Group Art Unit: 1637
	)	
Bert VOGELSTEIN et al	)	Prior Examiner: S. Woolwine
	)	
Serial No. 12/617,368	)	Confirmation No. 4461
	)	
Filed: November 12, 2009	)	Atty. Dkt. No. 001107.00794
	)	
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 September 16, 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 ~~mutated~~ vs. ~~one mutation in each of two alleles~~ both mutations in 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 to correct an obvious error and/or to improve clarity.

Respectfully submitted,

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

Date: October 13, 2010

Banner & Witcoff, Ltd.
Customer No. 22907

Electronic Acknowledgement Receipt

EFS ID:	8617961
Application Number:	12617368
International Application Number:	
Confirmation Number:	4461
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:	001107.00794
Receipt Date:	13-OCT-2010
Filing Date:	12-NOV-2009
Time Stamp:	15:11:19
Application Type:	Utility under 35 USC 111(a)

Payment information:

Submitted with Payment	no

File Listing:

Document Number	Document Description	File Name	File Size(Bytes)/ Message Digest	Multi Part /.zip	Pages (if appl.)
1	Supplemental Response or Supplemental Amendment	00794suppamd.pdf	90958 79eb7f6462763500d1b0b3f7abd2b253c17563ed	no	3

Warnings:	
Information:	

Total Files Size (in bytes):	90958

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
12/617,368	11/12/2009	

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 10/06/2010

	(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))	* 18	Minus	** 20	= 0	X $ =		OR	X $52=	0
Independent (37 CFR 1.16(h))	* 2	Minus	***3	= 0	X $ =		OR	X $220=	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 10/13/2010

	(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))	* 18	Minus	** 20	= 0	X $ =		OR	X $52 =	0
Independent (37 CFR 1.16(h))	* 2	Minus	*** 3	= 0	X $ =		OR	X $220 =	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

* 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:
/GLENN BURNS JR/

PATENT

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

Ins re Application of	)	Group Art Unit: 1637
	)	
Bert VOGELSTEIN et al	)	Examiner: S. Woolwine
	)	
Serial No. 12/617,368	)	Confirmation No. 4461
	)	
Filed: November 12, 2009	)	Atty. Dkt. No. 001107.00794
	)	
For: DIGITAL AMPLIFICATION	)	

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 non-final office action mailed September 23, 2010, Applicants submit and request that the Patent Office enter the claim amendment and the terminal disclaimer.

In the event that any fees or credits are due, please charge or credit our deposit account no. 19-0733.

IIN THE CLAIMS:

Please substitute the following claim set for those currently of record.

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

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

4. (Original) 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. (Original) 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. (Original) 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. (Original) The method of claim 1 wherein the sample is from blood.

8. (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 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. (Original) The method of claim 8 wherein the sample is from blood.

10. (Previously presented) The method of claim 1 or 8 wherein between 0.1 and 0.6 of the assay samples yield an amplification product.

11. (Previously presented) The method of claim 1 or 8 wherein between 0.3 and 0.5 of the assay samples yield an amplification product.

12. (Previously presented) The method of claim 1 or 8 wherein the set comprises at least 500 assay samples.

13. (Previously presented) The method of claim 1 or 8 wherein the set comprises at least 1000 assay samples.

14. (New) The method of claim 8 wherein the step of amplifying employs real-time polymerase chain reactions.

15. (New) The method of claim 14 wherein the real-time polymerase chain reactions comprise a dual-labeled fluorogenic probe.

16. (New) 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. (New) 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. (New) 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.

Remarks

New dependent claims on claim 8, claims 14-18, are supported *inter alia* by original dependent claims on claim 1, claims 2-6.

Applicant notes the reconsideration of the issue of new matter and appreciates the conclusion that the subject matter of claim 1 was disclosed in the earliest priority application as well as in the particular application as originally filed.

Claims 1 and 6-13 stand rejected for non-statutory double patenting over claims 3, 7-11, 19, 24, and 31 of parent patent U.S. 6,440,706. Similarly, claims 2 and 3 stand rejected over the same set of issued claims combined with claims 12 and 13 of the '706 patent and combined with the Marras literature reference. Applicants submit a terminal disclaimer over the '706 which obviates these rejections.

If all issues are resolved, we request that the U.S. Patent and Trademark Office process this application for grant.

Respectfully submitted,

Date: October 6, 2010

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

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) 001107.00794

In re Application of: VOGELSTEIN ET AL.

Application No.: 12/617,368

Filed: November 12, 2009

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
06 October 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:	12617368
Filing Date:	12-Nov-2009
Title of Invention:	Digital Amplification
First Named Inventor/Applicant Name:	BERT VOGELSTEIN
Filer:	Sarah Anne Kagan.
Attorney Docket Number:	001107.00794

Filed as Large Entity

Utility under 35 USC 111(a) Filing Fees

Description	Fee Code	Quantity	Amount	Sub-Total in USD($)
Basic Filing:				
Pages:				
Claims:				
Claims in excess of 20	1202	2	52	104
Miscellaneous-Filing:				
Petition:				
Patent-Appeals-and-Interference:				
Post-Allowance-and-Post-Issuance:				
Extension-of-Time:				

Description	Fee Code	Quantity	Amount	Sub-Total in USD($)
Miscellaneous:				
Statutory disclaimer	1814	1	140	140
		Total in USD ($)		**244**

Electronic Acknowledgement Receipt

EFS ID:	8569418
Application Number:	12617368
International Application Number:	
Confirmation Number:	4461
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:	001107.00794
Receipt Date:	06-OCT-2010
Filing Date:	12-NOV-2009
Time Stamp:	12:19:25
Application Type:	Utility under 35 USC 111(a)

Payment information:

Submitted with Payment	yes
Payment Type	Deposit Account
Payment was successfully received in RAM	$244
RAM confirmation Number	8687
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	Amendment/Req. Reconsideration-After Non-Final Reject	amd00794.pdf	89216 60fb088fafc668ab8017ba89a34194fac3391089	no	5
Warnings:					
Information:					
2	Terminal Disclaimer Filed	td00794.pdf	176452 f7afe3b202d4b8e6fd4168e8da7dd331ac4a40ff	no	2
Warnings:					
Information:					
3	Fee Worksheet (PTO-875)	fee-info.pdf	31794 354fa0b43d3e278ddcd66bcdc85e6897815d0a35	no	2
Warnings:					
Information:					
			Total Files Size (in bytes):	297462	

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
12/617,368	11/12/2009	

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 10/06/2010	(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))	* 18	Minus	** 20	= 0	X $ =		OR	X $52=	0
Independent (37 CFR 1.16(h))	* 2	Minus	***3	= 0	X $ =		OR	X $220=	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:
/NINA RATANAVONG/

UNITED STATES PATENT AND TRADEMARK OFFICE

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APPLICATION NO.	FILING DATE	FIRST NAMED INVENTOR	ATTORNEY DOCKET NO.	CONFIRMATION NO.
12/617,368	11/12/2009	BERT VOGELSTEIN	001107.00794	4461

22907 7590 09/23/2010

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
09/23/2010	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)	
	12/617,368	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 _____.

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) *1-13* 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) *1-3 and 6-13* is/are rejected.

7)☒ Claim(s) *4 and 5* 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 :11/12/2009;06/25/2010;09/16/2010.

DETAILED ACTION

Priority

This application is a continuation of application serial number 11/709,742. Instant claim 1 is identical to a claim that was rejected in the '742 application under 35 USC 112, 1st paragraph as "new matter" (see Office action mailed 06/11/2010 in the file for the '742 application; rejection of claim 45). The Examiner here reconsiders whether there was in fact support for this claim in the parent application, and further in the first non-provisional application in this series, i.e. application serial number 09/613,826 (now U.S. Patent No. 6,440,706).

Claim 1 of the instant application is a method for "determining an allelic imbalance" in a sample, wherein one determines "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", and comparing the first and second numbers to ascertain an allelic imbalance in the sample.

Looking at the Table 1 of the '706 patent, it is seen that "allelic imbalance" is one of the applications given for digital PCR (Dig-PCR). However, this table clearly indicates a technique where "allelic imbalance" is analyzed by "quantitative analysis with non-polymorphic markers" using a first probe that detects a marker sequence, and a second probe that detects a marker from another chromosome. This cannot support instant claim 1, since there would not be a "first allelic form of a marker" and a "second allelic form of the marker" for markers that are "non-polymorphic".

However, looking elsewhere in the '706 patent, it is clearly disclosed to determine "the ratio of a selected genetic sequence in a population of genetic sequences" by amplifying template molecules within a set of assay samples, determining a first number of assay samples containing the selected genetic sequence, determining a second number of assay samples containing a reference genetic sequence, and comparing the first number and second number to ascertain a ratio which reflects the composition of the biological sample (column 2, lines 18-30). The '706 patent also clearly states that allelic imbalances can be detected using digital amplification (column 6, lines 43-44).

Aside from these particular passages, it is noted that there is inherent support for instant claim 1 based on the following:

At column 3, lines 15-19, it is disclosed: "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." It is noted that "WT" is one allelic form of the marker, whereas Gly12Ser is another allelic form of the marker (similarly, the tumor harboring the Gly12Asp mutation also has two allelic forms: one WT and one mutant). It is stated in this passage that the tumors are aneuploid, having two or more alleles [i.e. copies] of the mutant allele for each allele [copy] of the WT allele. This is an allelic imbalance. In this passage, the allelic imbalance was not determined according to the claim (i.e. by comparing a "first number" of samples and a "second number" of samples). Rather, it was apparently known each of these tumors was aneuploid, and this was reflected in the ratio of red

and green signals (red from a probe detecting the PCR product whether mutant or WT; green from a probe detecting preferentially WT; see column 3, lines 1-4).

However, in figure 4, which shows the outcome of the digital PCR assay for the Gly12Asp sample (which as established above has an allelic imbalance with more copies of the mutant allele than the WT allele), it can be seen that the number of assay samples having the mutant allele (indicated by a red/green ration >3.0; see column 3, lines 35-45) is twice the number of assay samples having the WT allele (indicated by a red/green ratio ~1.0), which agrees with the earlier determination at column 3, lines 15-19 that this tumor sample had 2 or more copies of mutant allele per WT allele.

Following up on this analysis, at column 12, lines 1-5, describing the experiment shown in figure 4, it is disclosed: "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)." The reference to figure 2 is a reference to an experiment where the various tumor samples had been subjected to sequencing analysis, and where it was concluded that the Gly12Ser and Gly12Asp tumors where aneuploid (column 3, lines 5-20). However, the statement at column 12, lines 1-5 clearly indicates a ratio of WT to mutant alleles that is based on the number of assay samples containing each, which is precisely in accordance with instant claim 1.

Based on the above, the Examiner finds support for the instant claims in the originally filed non-provisional application serial number 09/613,826, and given that the

same disclosure is found in the provisional application 60/146,792, finds the claims entitled to a priority date of 08/02/1999.

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 1 and 6-13 are rejected on the ground of nonstatutory obviousness-type double patenting as being unpatentable over claims 3, 7-11, 19 and 31, and claim 24 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.

Claim 3 of the '706 patent discloses instant claims 1 and 8 except for the limitations that the selected genetic sequence is a first allelic form of a marker and the reference sequence is a second allelic form of a marker, and that the method

determines an allelic imbalance. However, claim 31 of the '706 patent discloses that the selected genetic sequence comprises a first mutation and the reference sequence comprises a second mutation, and claim 19 of the '706 patent discloses amplification with the same set of primers, implicitly disclosing that the selected genetic sequence and the reference sequence are the same amplifiable sequence. Since mutations are "allelic forms" of markers, the combined disclosures of claims 3, 19 and 31 of the '706 patent render instant claims 1 and 8 obvious, since the "ratio" (see claim 1 of the '706 patent, which limitations are present in claim 3 by dependency) inherently ascertains an allelic imbalance in the context of claim 31 of the '706 patent.

With regard to instant claim 6, it is considered that a dilution resulting in 0.1 of the assay samples having an amplification product would inherently result in homogeneity, based on the statement made at page 10, 2nd full paragraph of the instant specification: "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." If there is only a single template molecule per assay sample, then the amplification would inherently produce a homogeneous amplification product.

With regard to instant claims 7 and 9, claim 24 of the '706 patent discloses blood.

With regard to instant claims 10 and 11, 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 12 and 13, issued claims 10 and 11 disclose the number of assay samples is greater than 500, or greater than 1000.

Claims 2 and 3 are rejected on the ground of nonstatutory obviousness-type double patenting as being unpatentable over claims 3, 7-11, 19 and 31, and claim 24 of U.S. Patent No. 6,440,706 as applied to instant claims 1 and 6-13 above, and further in view of claims 12 and 13 of the '706 patent and Marras et al (Genetic Analysis: Biomolecular Engineering 14:151-156, Feb 1999, cited on the IDS of 11/12/2009).

The disclosure of claims 3, 7-11, 19 and 31 of the '706 patent have been discussed. In addition, claim 13 of the '706 patent discloses using molecular beacon probes for the analysis, and claim 12 of the '706 patent discloses that the amplifying and analyzing steps are conducted in the same receptacle (e.g. tube).

The claims of the '706 patent, then, disclose amplifying and analyzing with dual-labeled fluorogenic probes, but do not disclose "real-time polymerase chain reaction" as recited in claim 2.

Marras disclosed real-time PCR using molecular beacons for multiplex determination of single-nucleotide polymorphisms (see entire article, especially section 3.4 on page 154).

It would have been *prima facie* obvious to one of ordinary skill in the art at the time the invention was made to carry out the amplification and molecular beacon analysis disclosed in the claims of the '706 patent in a real-time PCR as taught by

Marras, because this would have been faster than conducting the PCR and molecular beacon analysis in separate steps.

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/
Primary Examiner

Search Notes	Application/Control No.	Applicant(s)/Patent Under Reexamination
12617368	12617368	VOGELSTEIN ET AL.
	Examiner	Art Unit
	SAMUEL C WOOLWINE	1637

SEARCHED			
Class	Subclass	Date	Examiner

SEARCH NOTES		
Search Notes	Date	Examiner
Inventor name, keyword search in EAST, Google Scholar (see printouts); review prosecution history of parent applications; consideration of IDSs.	09/21/2010	SCW

INTERFERENCE SEARCH			
Class	Subclass	Date	Examiner

INFORMATION DISCLOSURE STATEMENT BY APPLICANT (**Not for submission under 37 CFR 1.99)**		
	Application Number	
	Filing Date	2009-11-06
	First Named Inventor	Bert Vogelstein et al.
	Art Unit	TBD
	Examiner Name	TBD
	Attorney Docket Number	001107.00794

U.S.PATENTS						
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
/S.W./	1	5213961		1993-05-25	Bunn et al.	
/S.W./	2	5736333		1998-04-07	Livak et al.	
/S.W./	3	5518901		1996-05-21	Murtagh	
/S.W./	4	5804383		1998-09-08	Gruenert et al.	
/S.W./	5	5858663		1999-01-12	Nisson et al.	
/S.W./	6	5670325		1997-09-23	Lapidus et al.	
/S.W./	7	6037130		2000-03-14	Tyagi et al.	
/S.W./	8	5925517		1999-07-20	Tyagi et al.	

INFORMATION DISCLOSURE STATEMENT BY APPLICANT (Not for submission under 37 CFR 1.99)		
	Application Number	
	Filing Date	2009-11-06
	First Named Inventor	Bert Vogelstein et al.
	Art Unit	TBD
	Examiner Name	TBD
	Attorney Docket Number	001107.00794

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

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

U.S.PATENT APPLICATION PUBLICATIONS

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.

FOREIGN PATENT DOCUMENTS

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]
/S.W./	1	95/13399	WO		1995-05-18			☐
/S.W./	2	99/13113	WO		1999-03-18			☐

INFORMATION DISCLOSURE STATEMENT BY APPLICANT (Not for submission under 37 CFR 1.99)		
	Application Number	
	Filing Date	2009-11-06
	First Named Inventor	Bert Vogelstein et al.
	Art Unit	TBD
	Examiner Name	TBD
	Attorney Docket Number	001107.00794

/S.W./	3	0643140	EP		1995-03-15			☐

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

NON-PATENT LITERATURE DOCUMENTS

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]
/S.W./	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	☐
/S.W./	2	P. J. SYKES, "Quantitation of Targets for PCR by Use of Limiting Dilution," BioTechniques, 1992, Vol. 13, No. 3, pp. 444-449	☐
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	Application Number	
	Filing Date	2009-11-06
	First Named Inventor	Bert Vogelstein et al.
	Art Unit	TBD
	Examiner Name	TBD
	Attorney Docket Number	001107.00794

/S.W./	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	☐
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	First Named Inventor	Bert Vogelstein et al.
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	Examiner Name	TBD
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/S.W./	20	MARRAS ET AL., "Multiplex Detection of Single-Nucleotide Variations Using Molecular Beacons," Genetic Analysis: Biomolecular Engineering, Feb. 1999, Vo. 14, pp. 151-156	☐
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S2	7	S1 and (allele alleles allelic)	USPAT	OR	OFF	2010/09/16 09:06
S3	1	S1 and ((allele alleles allelic) near5 imbalance$1)	USPAT	OR	OFF	2010/09/16 09:07
S4	110	((loss adj2 heterozygosity) ((allele alleles allelic) near5 imbalance$1)) and (sample near5 (dilute diluted diluting dilution divide divided dividing split splitting portions aliquot aliquotted aliquotting aliquots)) near30 (pcr amplification amplified)	US-PGPUB; USPAT; EPO; JPO; DERWENT	OR	OFF	2010/09/16 09:16
S5	20	S4 and (@ad<"19990802" @pd<"19990802")	US-PGPUB; USPAT; EPO; JPO; DERWENT	OR	OFF	2010/09/16 09:17
S6	15	((loss adj2 heterozygosity) ((allele alleles allelic) near5 imbalance$1)).clm. and (dilute diluted diluting dilution divide divided dividing split splitting portions aliquot aliquotted aliquotting aliquots).clm. and (amplification pcr).clm.	US-PGPUB; USPAT; EPO; JPO; DERWENT	OR	OFF	2010/09/16 09:22

EAST Search History (Interference)

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S7	3	((loss adj2 heterozygosity) ((allele alleles allelic) near5 imbalance$1)).clm. and (dilute diluted diluting dilution divide divided dividing split splitting portions aliquot aliquotted aliquotting aliquots).clm. and (amplification pcr).clm.	USPAT; UPAD	OR	OFF	2010/09/16 09:29

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L3	14	(allel$2 near3 imbalance $1) and (number near3 (fractions times occurrences events occaisions occasions)) same (pcr amplification amplify amplifying amplified amplifies)	US-PGPUB; USPAT; USOCR; EPO; JPO; DERWENT	OR	OFF	2010/09/21 11:18
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L5	9	(allel$2 near3 imbalance $1) and (number near3 (samples aliquots portions)) near60 (pcr amplification amplify amplifying amplified amplifies)	US-PGPUB; USPAT; USOCR; EPO; JPO; DERWENT	OR	OFF	2010/09/21 11:19
L6	10	(allel$2 near3 imbalance $1) and (number near3 (samples aliquots portions)) near99 (pcr amplification amplify amplifying amplified amplifies)	US-PGPUB; USPAT; USOCR; EPO; JPO; DERWENT	OR	OFF	2010/09/21 11:19
L7	85	(allel$2 near3 imbalance $1) and (number near3 (samples aliquots portions)) and (pcr amplification amplify amplifying amplified amplifies)	US-PGPUB; USPAT; USOCR; EPO; JPO; DERWENT	OR	OFF	2010/09/21 11:20

L8	7	l7 and (@ad<"19990802" @pd<"19990802")	US-PGPUB; USPAT; USOCR; EPO; JPO; DERWENT	OR	OFF	2010/09/21 11:20
L9	7	l1 and (vogelstein vogelstien kinzler).in.	US-PGPUB; USPAT; USOCR; EPO; JPO; DERWENT	OR	OFF	2010/09/21 11:25
L10	1	(digital adj1 pcr) and (@ad<"19990802" @pd<"19990802")	US-PGPUB; USPAT; USOCR; EPO; JPO; DERWENT	OR	OFF	2010/09/21 11:33

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Application Number	12617368
Filing Date	2009-11-12
First Named Inventor	Bert VOGELSTEIN, et al.
Art Unit	1634
Examiner Name	
Attorney Docket Number	001107.00794

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	First Named Inventor	Bert VOGELSTEIN, et al.
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	Attorney Docket Number	001107.00794

/S.W./	1	Notice of Reasons for Rejection dispatched April 28, 2010 in Japanese Application No. 2001-513641 and English translation thereof.	☐
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Application Number	12617368
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First Named Inventor	VOGELSTEIN, BERT
Art Unit	1637
Examiner Name	WOOLWINE, SAMUEL
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	Attorney Docket Number	001107.00794

/S.W./	1	NEWTON, PCR Essential Data, pages 51-52, 1995	☐
/S.W./	2	Office Action dated June 11, 2010, in co-pending application 11709742	☐
/S.W./	3	Office Action dated December 29, 2009 in co-pending application 11709742	☐
/S.W./	4	Office Action dated September 18, 2009 in co-pending application 11709742	☐
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B Vogelstein, KW Kinzler - … of Sciences of the United States …, 1999 - National Acad Sciences
The identification of predefined mutations expected to be present in a minor fraction of a cell population is important for a variety of basic research and clinical applications. Here, we describe an approach for transforming the exponential, analog nature of the PCR into a linear, **...**
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RD Tarver, M Cohen, NJ Broderick, DJ … - Journal of thoracic …, 1990 - journals.lww.com
... chest radiographs. One of the most crucial portable chest observations that depends on high resolution is the detection of small pneumothora- ces (Fig 4). The **digital PCR** system performs this task well. The decreased resolution **...**
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B Vogelstein, KW Kinzler - Natl Acad. Sci. USA, 1999
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A direct digital control for the phase-controlled rectifier
EH Song, BH Kwon - IEEE Transactions on Industrial …, 1991 - ieeexplore.ieee.org
... VOL. 38, NO. 5, OCTOBER 1991 Intel 8797 single-chip microcomputer Fig. 1. System configuration of a current source using **digital PCR ...** Its specification is shown in Table I [20]. The function of the **digital PCR** can be divided into diagnosis, control, and pulse generation. **...**
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Quantitative abnormalities of fetal DNA in maternal serum in preeclampsia
YM Lo, TN Leung, MSC Tein, IL Sargent, J … - Clinical …, 1999 - Am Assoc Clin Chem
... Home page, Clin. Chem. Home page FMF Lun, RWK Chiu, KC Allen Chan, T. Yeung Leung, T. Kin Lau, and YM Dennis Lo Microfluidics **Digital PCR** Reveals a Higher than Expected Fraction of Fetal DNA in Maternal Plasma Clin. Chem., October 1, 2008; 54(10): 1664 - 1672. **...**
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NJ Gibson, HL Gillard, D Whitcombe, RM … - Clinical …, 1997 - Am Assoc Clin Chem
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H Grundmann, A Hahn, B Ehrenstein, … - Clinical …, 1999 - interscience.wiley.com
... The typing was performed by PCR fingerprinting using random amplification of polymorphic DNA (RAPD), coupled with automated laser fluorescence analysis (ALFA), which allows the generation of **digital PCR** fingerprint data of high accuracy [9]. With this approach, the strain **...**
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B Pathophysiology, M Benjamin, M Johns - Analysis, 1998 - cancer.emory.edu
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Loss of chromosome arm 8p loci in prostate cancer: mapping by quantitative **allelic imbalance**
D MacGrogan, A Levy, D Bostwick, M ... - Genes, ..., 1994 - interscience.wiley.com
... Statistical Analyses Distributions of **allelic imbalance** data, Student's test, and chi-square statistics were ... with microsatellite amplifica- tion in many previous studies; 2) smaller **allelic** bands were ... for **PCR**, yielding autora- diographic signals proportional to molar **numbers** of single ...
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Comparative genomic hybridization, **allelic imbalance**, and fluorescence in situ hybridization on chromosome 8 in prostate cancer
ML Cher, D Macgrogan, R Bookstein, ... - Genes, ..., 1994 - interscience.wiley.com
... Figure 3. Detailed comparison of CGH data with **allelic imbalance** data Informative **PCR** (top line) and ... An **allelic** index > I .5 is considered to be **allelic imbalance** (MacGrogan et al.. ... profiles are plotted as in Figure 2. Chromosomal regions of relative DNA sequence copy **number** ...
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Microsatellite instability and 8p **allelic imbalance** in stage B2 and C colorectal cancers — oxfordjournals.org [HTML]
KC Halling, AJ French, SK McDonnell, ... - JNCI Journal of the ..., 1999 - jnci.oxfordjournals.org
... Microsatellite Analysis. Tumors were analyzed for MSI and **allelic imbalance** at 11 dinucleotide microsatellite markers on chromosomes 5q, 8p, 15q, 17p, and 18q as previously described (25). ... The median **number** of markers that gave a **PCR** product for both normal DNA ...
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PCR-based microsatellite polymorphisms in the detection of loss of heterozygosity in fresh and archival tumour tissue. — nih.gov [PDF]
NA Gruis, EC Abeln, AF Bardoel, P Devilee, ... - British journal of ..., 1993 - ncbi.nlm.nih.gov
... of the **allelic imbalance** obtained after 28 cycles. There is a good agreement between the expected and observed **imbalance**-factors for each ratio after 28 cycles of amplification (Table II). Thus the **number** of cycles does not influence the outcome of the **PCR** reaction and does ...
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Allelic imbalance, including deletion of PTEN/MMAC1, at the Cowden disease locus on 10q22-23, in hamartomas from patients with Cowden syndrome and germline ...
DJ Marsh, PLM Dahia, V Coulon, Z ... - Genes, ..., 1998 - interscience.wiley.com
... Semi-quantitative **PCR** performed on RNA from hamartomas from three different tissues from a ... Charles A. Dana Foundation; and the American Cancer Society; Contract Grant **number**: RPG-97 ... **Allelic imbalance** or loss of heterozygosity (LOH) in the CD locus had been observed ...
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Allelic imbalance and microsatellite instability in prostatic adenocarcinoma — aacrjournals.org [PDF]
JM Cunningham, A Shan, MJ Wick, SK McDonnell, DJ ... - Cancer research, 1996 - AACR
... autopsy, frozen, or paraffin- embedded), the **number** of chromosomes examined, the **number** of markers ... mageanalysis were utili/ed, the criteria established for assessment of **imbalance** or loss ... **Allelic** loss on 8p occurs in hepa- tocellulur carcinoma (48), colorectal carcinoma (31. ...
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Allelotype of head and neck paragangliomas: **allelic imbalance** is confined to the long arm of chromosome 11, the site of the predisposing locus PGL
P Devilee, EM Van Schothorst, AFJ ... - Genes, ..., 1994 - interscience.wiley.com
... Gels containing **PCR** products were exposed overnight to a PhosphorImaging screen (Molecular Dynamics), and the **number** of counts ... We have previously defined the **allelic imbalance** factor (AIF) in the tumor as the ratio between these two ratios (Devilee et ...
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Search for mutations and examination of **allelic** expression **imbalance** of the p73 gene at 1p36. 33 in human lung cancers — aacrjournals.org [PDF]
S Nomoto, N Haruki, M Kondo, H Konishi, T Takahashi, ... - Cancer research, 1998 - AACR
... polymorphisms in the coding exons and the sequence variations in noncoding exon 2. Although p73 mutations could eventually be identified by means of selective screening of a larger **number** of cases ... Although marked **allelic** expression **imbalance** was observed ...
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At least two different regions are involved in **allelic imbalance** on chromosome arm 16q in breast cancer
AM Cleton-Jansen, EW Moerland, NJ ... - Genes, ..., 1994 - interscience.wiley.com
... Cases that were still ambiguous were rerun on a second Southern blot or **PCR** reaction. ... Page 3. ALLEUC **IMBALANCE** ON 169 IN BREAST CANCER TABLE I. **Allelic Imbalance** on Chromosome I6 in 79 Breast Carcinomas 103 Type **Number** of **Number** of **Number** of ...
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An allelotype of squamous carcinoma of the head and neck using microsatellite markers — aacrjournals.org [PDF]
KW Ah-See, TG Cocke, IR Pickford, D Soutar, A ... - Cancer research, 1994 - AACR

... for quantification of alÃ-elecopy **numbers** and that amplification by **PCR** is often dogged by "shadow" bands making interpretation occasionally difficult. Our results demonstrate that several chromosomal regions show a much higher frequency of **allelic** loss or **imbalance** than the ...
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Frequent genetic alterations at the distal region of chromosome 1p in human hepatocellular carcinomas aacrjournals.org [PDF]
SH Yeh, PJ Chen, HL Chen, MY Lai, CC Wang, DS . . - Cancer research, 1994 - AACR
... D1S57 D1S85 D1S57 D1S85 Fig. 2. **Allelic** loss and **imbalance** on chromosome ip as shown by **PCR** amplification of microsatellite marker D15186 (A) or by Southern hybridization with probe D1557 or D1585 (B). LOH is found in cases 15 and 20. **...**
Cited by 113 - Related articles - BL Direct - All 4 versions

Four regions of **allelic imbalance** on 17q12-qter associated with high-grade breast tumors
SJ Plummer, MJ Paris, J Myles, R ... - Genes, ..., 1997 - interscience.wiley.com
... Number of samples/total **number of samples** (percent) Histopathologic type DCISa 8/85 (10) Grade Ib 21/85 (25) Grade IIb 36 **...** Because these stud- ies were performed by **PCR**, **allelic imbalance** may represent DNA amplification or loss of heterozygos- ity (LOH) of chromosomal **...**
Cited by 44 - Related articles - BL Direct - All 2 versions

Allelic imbalance on chromosome I in human breast cancer. II. Microsatellite repeat analysis
N Hoggard, B Brintnell, J Varley, A ... - Genes, ..., 1995 - interscience.wiley.com
... Paired normal and tumour DNAs from each patient were analysed for **allelic imbalance** using microsatellite markers on chromo- some 1. A primary **PCR** was carried out on approx- imately 50 ng of genomic DNA in a total volume of 20 p1 of lxTaq polymerase buffer containing **...**
Cited by 54 - Related articles - BL Direct - All 3 versions

Allelic imbalance in gastric cancer: an affected site on chromosome arm 3p
BG Schneider, DR Pulitzer, RD Brown, ... - Genes, ..., 1995 - interscience.wiley.com
... analysis of formalin- fixed, paraffin-embedded archival specimens, greatly increasing the **number of samples** suitable for **...** Figure I. Summary of **allelic imbalance** (AI) analysis. **...** For detection of heterozygosity, **PCR** products were electrophoresed on formamide-urea denatur- ing **...**
Cited by 64 - Related articles - BL Direct - All 2 versions

Infrequent mutations of p27Kip1 gene and trisomy 12 in a subset of human pituitary adenomas endojournals.org [HTML]
C Tanaka, K Yoshimoto, P Yang, T Kimura, ... - Journal of Clinical ..., 1997 - Endocrine Soc
... The fluorescent **PCR** microsatellite analysis was shown eligible in this study to detect trisomy **...** Although the small **number of samples** in our study made it impossible to point out the **...** 1996 **Allelic imbalance** on chromosome 13q: evidence for the involvement of BRCA2 and RB1 in **...**
Cited by 58 - Related articles - BL Direct - All 5 versions

Somatic deletions and mutations in the Cowden disease gene, PTEN, in sporadic thyroid tumors aacrjournals.org [PDF]
PLM Dahia, DJ Marsh, Z Zheng, J Zedenius, P ... - Cancer research, 1997 - AACR
... **PCR** products were gel and column purified using the Wizard **PCR**-Prep kit **...** Analysis of a larger **number of samples**, including benign tumors, may help clarify whether qualitative or **...** AL., Robinson, BG, Weber, HC, Longy, M., and Eng, C. **Allelic imbalance**, including deletion of **...**
Cited by 197 - Related articles - BL Direct - All 3 versions

Different alleles cause an **imbalance** in A2 and A2B phenotypes of the ABO blood group
K Ogasawara, R Yabe, M Uchikawa, M ... - Vox ..., 1998 - interscience.wiley.com
... B al- lele could not be discriminated from the common *B101 in the present **PCR**-SSCP analysis **...** These observations suggest that the **imbalance** between the A2B/AjB ratio and the A2/A, ratio in **...** This **allele** has a unique property in that it ac- counts for both Aj and A2 phenotypes **...**
Cited by 49 - Related articles - All 4 versions

Microsatellite analysis of endometriosis reveals loss of heterozygosity at candidate ovarian tumor suppressor gene loci aacrjournals.org [PDF]
X Jiang, A Hitchcock, EJ Bryan, RH Watson, P ... - Cancer research, 1996 - AACR
... However, the impact of this study is limited by both the small **number of samples** examined and **...** 5. **Allelic** deletion for endometrioid ovarian cancer OC95 and endometriosis cases E7, E23, and **...** for each informative locus, the autoradiography of the **PCR** result from the normal (N **...**
Cited by 124 - Related articles - BL Direct - All 3 versions

Allelic loss of 16q23. 2-24.2 is an independent marker of good prognosis in primary breast cancer aacrjournals.org [PDF]
LL Hansen, M Yilmaz, J Overgaard, J Andersen, TA ... - Cancer research, 1998 - AACR
... 22. **PCR** amplification of tumor and normal DNA was carried out in microtiter wells as **...** of tumors biased toward good prognosis, inherited breast cancer, and the limited **number of samples**. **...** S. High frequency of **allelic imbalance** at chromosome region l6q22-23 in human breast **...**
Cited by 46 - Related articles - BL Direct - All 3 versions

Genetically abnormal clones in histologically normal breast tissue. nih.gov [PDF]
PS Larson, A De las Morenas, LA Cupples ... - The American journal ..., 1998 - ncbi.nlm.nih.gov
... of the limited quantities of DNA available, unequal amplification in early **PCR** cycles could **...** at heterozygous loci based on densitometry were not calculated, and relative **allele imbalance** was scored **...** **allelic** alterations should usually affect at least a substan- tial fraction of the cells **...**
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PATENT

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re Application of	)	Prior Group Art Unit: 1637
	)	
Bert VOGELSTEIN et al	)	Prior Examiner: S. Woolwine
	)	
Serial No. 12/617,368	)	Confirmation No. 4461
	)	
Filed: November 12, 2009	)	Atty. Dkt. No. 001107.00794
	)	
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 Patent Office enter the amendment to the application and consider the information disclosure statement filed concurrently.

IN THE SPECIFICATION

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 Fl 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, Ell, 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 Fl: 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-Dabcy1-3' (SEQ ID NO: 4); MB-GREEN: 5'-Fluorescein-CACGGGAGCTGGTGGCGTAGCGTG-Dabcy1-3' (SEQ ID NO: 5). Molecular Beacons (33,34) were synthesized by Midland Scientific and other oligonucleotides were synthesized by Gene Link (Thornwood, NY). All were dissolved at 50 uM in TE (10 mM Tris, pH 8.0/ 1 mM EDTA) and kept frozen and in the dark until use. PCR products were purified using QIAquick PCR purification kits (Qiagen). In the relevant experiments described in the text, 20% of the product from single wells was used for gel electrophoresis and 40% was used for each sequencing reaction. The primer used for sequencing was 5'-CATTATTTTATTATAAGGCCTGC-3' (SEQ ID NO: 6). Sequencing was performed using fluorescently-labeled ABI Big Dye terminators and an ABI 377 automated sequencer.

Remarks

The amendments are to comply with the sequence rules and the rules for drawings.

Respectfully submitted,

Date: September 16, 2010

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

Banner & Witcoff, Ltd.
Customer No. 22907

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

(Not for submission under 37 CFR 1.99)

Application Number	12617368
Filing Date	2009-11-12
First Named Inventor	VOGELSTEIN, BERT
Art Unit	1637
Examiner Name	WOOLWINE, SAMUEL
Attorney Docket Number	001107.00794

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INFORMATION DISCLOSURE STATEMENT BY APPLICANT (Not for submission under 37 CFR 1.99)		
	Application Number	12617368
	Filing Date	2009-11-12
	First Named Inventor	VOGELSTEIN, BERT
	Art Unit	1637
	Examiner Name	WOOLWINE, SAMUEL
	Attorney Docket Number	001107.00794

	1	NEWTON, PCR Essential Data, pages 51-52, 1995	☐
	2	Office Action dated June 11, 2010, in co-pending application 11709742	☐
	3	Office Action dated December 29, 2009 in co-pending application 11709742	☐
	4	Office Action dated September 18, 2009 in co-pending application 11709742	☐
	5	Office Action dated June 5, 2009 in co-pending application 11709742	☐

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INFORMATION DISCLOSURE STATEMENT BY APPLICANT (Not for submission under 37 CFR 1.99)		
	Application Number	12617368
	Filing Date	2009-11-12
	First Named Inventor	VOGELSTEIN, BERT
	Art Unit	1637
	Examiner Name	WOOLWINE, SAMUEL
	Attorney Docket Number	001107.00794

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☐ 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)	2010-09-16
Name/Print	Sarah A. Kagan	Registration Number	32141

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Electronic Acknowledgement Receipt

EFS ID:	8431520
Application Number:	12617368
International Application Number:	
Confirmation Number:	4461
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:	001107.00794
Receipt Date:	16-SEP-2010
Filing Date:	12-NOV-2009
Time Stamp:	13:20:17
Application Type:	Utility under 35 USC 111(a)

Payment information:

Submitted with Payment	no

File Listing:

Document Number	Document Description	File Name	File Size(Bytes)/ Message Digest	Multi Part /.zip	Pages (if appl.)
1	Preliminary Amendment	prelimamd00794.pdf	80950 771a5c2df45b9e96debd7bb85b16b6610ce48b35	no	5

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PATENT APPLICATION FEE DETERMINATION RECORD

Substitute for Form PTO-875

Application or Docket Number	Filing Date	☐ To be Mailed
12/617,368	11/12/2009	

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))							
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APPLICATION AS AMENDED – PART II

AMENDMENT	09/16/2010	(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))	* 17	Minus	** 20	= 0	X $ =		OR	X $52=	0
	Independent (37 CFR 1.16(h))	* 2	Minus	***3	= 0	X $ =		OR	X $220=	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

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Legal Instrument Examiner:
/STEPHEN HOOVER/

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).

IDC-a2,AMD,M

~~Please replace the paragraph beginning page 6, line 1.~~

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.

IDC-a3,AMD

~~Please replace the paragraph beginning page 6, line 9.~~

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),

IDC-a4,AMD,M

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United States Patent and Trademark Office
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APPLICATION NUMBER	FILING OR 371(C) DATE	FIRST NAMED APPLICANT	ATTY. DOCKET NO./TITLE
12/617,368	11/12/2009	BERT VOGELSTEIN	001107.00794

CONFIRMATION NO. 4461

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

PUBLICATION NOTICE

OC000000043116717

Title: Digital Amplification

Publication No.US-2010-0209921-A1
Publication Date:08/19/2010

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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	12617368
Filing Date	2009-11-12
First Named Inventor	Bert VOGELSTEIN, et al.
Art Unit	1634
Examiner Name	
Attorney Docket Number	001107.00794

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INFORMATION DISCLOSURE STATEMENT BY APPLICANT (Not for submission under 37 CFR 1.99)		
	Application Number	12617368
	Filing Date	2009-11-12
	First Named Inventor	Bert VOGELSTEIN, et al.
	Art Unit	1634
	Examiner Name	
	Attorney Docket Number	001107.00794

	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).	☐

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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	12617368
	Filing Date	2009-11-12
	First Named Inventor	Bert VOGELSTEIN, et al.
	Art Unit	1634
	Examiner Name	
	Attorney Docket Number	001107.00794

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

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Electronic Acknowledgement Receipt

EFS ID:	7898604
Application Number:	12617368
International Application Number:	
Confirmation Number:	4461
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:	001107.00794
Receipt Date:	25-JUN-2010
Filing Date:	12-NOV-2009
Time Stamp:	18:10:17
Application Type:	Utility under 35 USC 111(a)

Payment information:

Submitted with Payment	no

File Listing:

Document Number	Document Description	File Name	File Size(Bytes)/ Message Digest	Multi Part /.zip	Pages (if appl.)
1	Information Disclosure Statement (IDS) Filed (SB/08)	IDS_SB08_off_JPOA_dtd_04_28_2010.PDF	612309 cca9e3ae51b0fcc050de09e3cf21855918c218dd	no	4

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A U.S. Patent Number Citation or a U.S. Publication Number Citation is required in the Information Disclosure Statement (IDS) form for autoloading of data into USPTO systems. You may remove the form to add the required data in order to correct the Informational Message if you are citing U.S. References. If you chose not to include U.S. References, the image of the form will be processed and be made available within the Image File Wrapper (IFW) system. However, no data will be extracted from this form. Any additional data such as Foreign Patent Documents or Non Patent Literature will be manually reviewed and keyed into USPTO systems.

2	NPL Documents	Notice_of_Reasons_for_Rejection_dtd_04_28_2010_JP2001-513641.PDF	222780 ea8cc419c94bbf201080ec2cfb009ca3de422506	no	6
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Information:					
3	NPL Documents	Stephens_et_al_TheoreticalUnderpinning_SingleMoleculeDilutionMethod_DirectHaplotypeResolution.PDF	1153082 f803d55f35cceb559ba91700b0f36a7d3655912e	no	7
Warnings:					
Information:					
		Total Files Size (in bytes):	1988171		

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APPLICATION NUMBER	FILING or 371(c) DATE	GRP ART UNIT	FIL FEE REC'D	ATTY.DOCKET.NO	TOT CLAIMS	IND CLAIMS
12/617,368	11/12/2009	1634	1480	001107.00794	13	2

CONFIRMATION NO. 4461

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

FILING RECEIPT

OC00000041567144

Date Mailed: 05/12/2010

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Applicant(s)

BERT VOGELSTEIN, BALTIMORE, MD;
KENNETH W. KINZLER, BALTIMORE, MD;

Assignment For Published Patent Application

THE JOHNS HOPKINS UNIVERSITY, BALTIMORE, MD

Power of Attorney:

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

Domestic Priority data as claimed by applicant

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

Foreign Applications

If Required, Foreign Filing License Granted: 05/06/2010

The country code and number of your priority application, to be used for filing abroad under the Paris Convention, is **US 12/617,368**

Projected Publication Date: 08/19/2010

Non-Publication Request: No

Early Publication Request: No

Title

Digital Amplification

Preliminary Class

435

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Electronic Acknowledgement Receipt

EFS ID:	6443895
Application Number:	12617368
International Application Number:	
Confirmation Number:	4461
05/11/2010 MTEKLEMI 00000004 190733 12617368 01 FC:1203 390.00 DA **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:	001107.00794
Receipt Date:	12-NOV-2009
Filing Date:	
Time Stamp:	16:49:10
Application Type:	Utility under 35 USC 111(a)

Payment information:

Submitted with Payment	yes
Payment Type	Deposit Account
Payment was successfully received in RAM	$1090
RAM confirmation Number	3585
Deposit Account	190733
Authorized User	
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Application Serial Number: 12617368
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Kinzler, Kenneth W.

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PATENT

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re Application of	)	Prior Group Art Unit: 1637
	)	
Bert VOGELSTEIN et al	)	Prior Examiner: S. Woolwine
	)	
Serial No. 12/617,368	)	Confirmation No. 4461
	)	
Filed: November 12, 2009	)	Atty. Dkt. No. 001107.00794
	)	
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 Patent Office enter the amendment to the claims prior to examination.

IN THE CLAIMS:

Please substitute the following set of claims for those currently of record:

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

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

4. (Original) 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. (Original) 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. (Original) 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. (Original) The method of claim 1 wherein the sample is from blood.

8. (Currently amended) 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~~ first allelic form of a marker and a second number of assay samples which contain a ~~reference genetic sequence on a second chromosome~~ 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 ~~chromosome~~ allelic form and the second ~~chromosome~~ allelic form in the biological sample.

9. (Original) The method of claim 8 wherein the sample is from blood.

10. (New) The method of claim 1 or 8 wherein between 0.1 and 0.6 of the assay samples yield an amplification product.

11. (New) The method of claim 1 or 8 wherein between 0.3 and 0.5 of the assay samples yield an amplification product.

12. (New) The method of claim 1 or 8 wherein the set comprises at least 500 assay samples.

13. (New) The method of claim 1 or 8 wherein the set comprises at least 1000 assay samples.

Remarks

This amendment corrects a clerical error. The claims in this divisional application were supposed to be directed to group II claims from the parent application. Inadvertently one of the claims from group I of the parent was presented in this application. No claims 10-13 were presented in the parent application and also formed part of group II. Claims 10 and 11 are supported at page 10, second full paragraph. Claims 12 and 13 are supported at page 10, third full paragraph. No new matter is added by this amendment.

Respectfully submitted,

Date: 18 March 2010

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

Banner & Witcoff, Ltd.
Customer No. 22907

Electronic Acknowledgement Receipt

EFS ID:	7240897
Application Number:	12617368
International Application Number:	
Confirmation Number:	4461
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:	001107.00794
Receipt Date:	18-MAR-2010
Filing Date:	
Time Stamp:	18:18:00
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	00794prelim.pdf	73819 21c9e672a1a4a68d7aac2825a9844b7e9274a1b3	no	5

Warnings:	
Information:	

Total Files Size (in bytes):	73819

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New Applications Under 35 U.S.C. 111

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

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

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

New International Application Filed with the USPTO as a Receiving Office

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

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

MULTIPLE DEPENDENT CLAIM FEE CALCULATION SHEET

Substitute for Form PTO-1360
(For use with Form PTO/SB/06)

Application Number	Filing Date
12/617,368	03/18/2010

Applicant(s)

* May be used for additional claims or amendments

CLAIMS	AS FILED		AFTER FIRST AMENDMENT		AFTER SECOND AMENDMENT		*			*			*		
	Indep	Depend	Indep	Depend	Indep	Depend		Indep	Depend		Indep	Depend		Indep	Depend
1	1						51								
2		1					52								
3		1					53								
4		1					54								
5		1					55								
6		1					56								
7		1					57								
8	1						58								
9		1					59								
10		2					60								
11		2					61								
12		2					62								
13		2					63								
14							64								
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43							93								
44							94								
45							95								
46							96								
47							97								
48							98								
49							99								
50							100								
Total CLAIMS	13						Total Indep								
Total CHARG.	17						Total Depend								
Total INDEP	2						Total Claims								

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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PTO/SB/06 (10-07)
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U.S. Patent and Trademark Office; U.S. DEPARTMENT OF COMMERCE

Date: 03/18/2010

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PATENT APPLICATION FEE DETERMINATION RECORD Substitute for Form PTO-875	Application or Docket Number 12/617,368

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	330
SEARCH FEE (37 CFR 1.16(k), (i), or (m))	N/A	N/A	N/A			N/A	540
EXAMINATION FEE (37 CFR 1.16(o), (p), or (q))	N/A	N/A	N/A			N/A	220
TOTAL CLAIMS (37 CFR 1.16(i))	17	*	X 26=		OR	X 52=	
INDEPENDENT CLAIMS (37 CFR 1.16(h))	2	*	X 110=			X 220=	
APPLICATION SIZE FEE (37 CFR 1.16(s))	If the specification and drawings exceed 100 sheets of paper, the application size fee due is $270 ($135 for small entity) for each additional 50 sheets or fraction thereof. See 35 U.S.C. 41(a)(1)(G) and 37 CFR						
MULTIPLE DEPENDENT CLAIM PRESENT (37 CFR 1.16(j))			195			390	390
* If the difference in column 1 is less than zero, enter "0" in column 2.			TOTAL			TOTAL	1480

APPLICATION AS AMENDED – PART II

		(Column 1)		(Column 2)	(Column 3)	SMALL ENTITY		OR	OTHER THAN SMALL ENTITY	
		CLAIMS REMAINING AFTER AMENDMENT		HIGHEST NUMBER PREVIOUSLY PAID FOR	PRESENT EXTRA	RATE ($)	ADDI-TIONAL FEE ($)		RATE ($)	ADDI-TIONAL FEE ($)
AMENDMENT A	Total (37 CFR 1.16(i))	*	Minus	**	=	X =		OR	X =	
	Independent (37 CFR 1.16(h))	*	Minus	***	=	X =		OR	X =	
	Application Size Fee (37 CFR 1.16(s))									
	FIRST PRESENTATION OF MULTIPLE DEPENDENT CLAIM (37 CFR 1.16(j))					N.A		OR	N/A	
						TOTAL ADD'T FEE		OR	TOTAL ADD'T FEE	

		(Column 1)		(Column 2)	(Column 3)			OR		
		CLAIMS REMAINING AFTER AMENDMENT		HIGHEST NUMBER PREVIOUSLY PAID FOR	PRESENT EXTRA	RATE ($)	ADDI-TIONAL FEE ($)		RATE ($)	ADDI-TIONAL FEE ($)
AMENDMENT B	Total (37 CFR 1.16(i))	*	Minus	**	=	X =		OR	X =	
	Independent (37 CFR 1.16(h))	*	Minus	***	=	X =		OR	X =	
	Application Size Fee (37 CFR 1.16(s))									
	FIRST PRESENTATION OF MULTIPLE DEPENDENT CLAIM (37 CFR 1.16(j))					N/A		OR	N/A	
						TOTAL ADD'T FEE		OR	TOTAL ADD'T 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.

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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
12/617,368	11/12/2009	

APPLICATION AS FILED – PART I

FOR	(Column 1) NUMBER FILED	(Column 2) NUMBER EXTRA	SMALL ENTITY RATE ($)	SMALL ENTITY FEE ($)	OR	OTHER THAN SMALL ENTITY RATE ($)	OTHER THAN SMALL ENTITY FEE ($)
☐ BASIC FEE (37 CFR 1.16(a), (b), or (c))	N/A	N/A	N/A			N/A	
☐ SEARCH FEE (37 CFR 1.16(k), (i), or (m))	N/A	N/A	N/A			N/A	
☐ EXAMINATION FEE (37 CFR 1.16(o), (p), or (q))	N/A	N/A	N/A			N/A	
TOTAL CLAIMS (37 CFR 1.16(i))	minus 20 =	*	X $ =		OR	X $ =	
INDEPENDENT CLAIMS (37 CFR 1.16(h))	minus 3 =	*	X $ =			X $ =	
☐ APPLICATION SIZE FEE (37 CFR 1.16(s))	If the specification and drawings exceed 100 sheets of paper, the application size fee due is $250 ($125 for small entity) for each additional 50 sheets or fraction thereof. See 35 U.S.C. 41(a)(1)(G) and 37 CFR 1.16(s).						
☐ MULTIPLE DEPENDENT CLAIM PRESENT (37 CFR 1.16(j))							
* If the difference in column 1 is less than zero, enter "0" in column 2.			TOTAL			TOTAL	

APPLICATION AS AMENDED – PART II

AMENDMENT 03/18/2010	(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))	* 17	Minus	** 20	= 0	X $ =		OR	X $52=	0
Independent (37 CFR 1.16(h))	* 2	Minus	***3	= 0	X $ =		OR	X $220=	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:
/SHANDA ROSS/

PATENT

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re Application of	)	Prior Group Art Unit: 1637
	)	
Bert VOGELSTEIN et al	)	Prior Examiner: Samuel Woolwine
	)	
Divisional Application of	)	Confirmation No. TBD
Serial No. 11/709,742	)	
	)	Atty. Dkt. No. 001107.00794
Filed: Herewith	)	
	)	
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, enclosed is a PTO Form 1449 listing documents for consideration by the Examiner in the subject application. Copies of the cited references were submitted in parent Application No. 11/709,742 or were provided by the Examiner attached to an office action. No fee is believed to be due to ensure consideration and entry of the cited documents by the Examiner. However, if a fee is deemed necessary, the Commissioner is authorized to charge our Deposit Account No. 19-0733.

Respectfully submitted,

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

Date: November 11, 2009

Banner & Witcoff, Ltd.
Customer No. 22907

PTO/SB/08a (05-07)
Approved for use through 11/30/2007. 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	
Filing Date	2009-11-06
First Named Inventor	Bert Vogelstein et al.
Art Unit	TBD
Examiner Name	TBD
Attorney Docket Number	001107.00794

U.S.PATENTS

Examiner Initial*	Cite No	Patent Number	Kind Code[1]	Issue Date	Name of Patentee or Applicant of cited Document	Pages,Columns,Lines where Relevant Passages or Relevant Figures Appear
	1	5213961		1993-05-25	Bunn et al.	
	2	5736333		1998-04-07	Livak et al.	
	3	5518901		1996-05-21	Murtagh	
	4	5804383		1998-09-08	Gruenert et al.	
	5	5858663		1999-01-12	Nisson et al.	
	6	5670325		1997-09-23	Lapidus et al.	
	7	6037130		2000-03-14	Tyagi et al.	
	8	5925517		1999-07-20	Tyagi et al.	

INFORMATION DISCLOSURE STATEMENT BY APPLICANT (Not for submission under 37 CFR 1.99)		
	Application Number	
	Filing Date	2009-11-06
	First Named Inventor	Bert Vogelstein et al.
	Art Unit	TBD
	Examiner Name	TBD
	Attorney Docket Number	001107.00794

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

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U.S.PATENT APPLICATION PUBLICATIONS

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

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

INFORMATION DISCLOSURE STATEMENT BY APPLICANT (Not for submission under 37 CFR 1.99)		
	Application Number	
	Filing Date	2009-11-06
	First Named Inventor	Bert Vogelstein et al.
	Art Unit	TBD
	Examiner Name	TBD
	Attorney Docket Number	001107.00794

	3	0643140	EP		1995-03-15			☐

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

NON-PATENT LITERATURE DOCUMENTS

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

INFORMATION DISCLOSURE STATEMENT BY APPLICANT (Not for submission under 37 CFR 1.99)		
	Application Number	
	Filing Date	2009-11-06
	First Named Inventor	Bert Vogelstein et al.
	Art Unit	TBD
	Examiner Name	TBD
	Attorney Docket Number	001107.00794

	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	☐

INFORMATION DISCLOSURE STATEMENT BY APPLICANT (Not for submission under 37 CFR 1.99)		
	Application Number	
	Filing Date	2009-11-06
	First Named Inventor	Bert Vogelstein et al.
	Art Unit	TBD
	Examiner Name	TBD
	Attorney Docket Number	001107.00794

	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	☐

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

EXAMINER SIGNATURE

Examiner Signature		Date Considered	

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

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

INFORMATION DISCLOSURE STATEMENT BY APPLICANT (**Not for submission under 37 CFR 1.99)**		
	Application Number	
	Filing Date	2009-11-06
	First Named Inventor	Bert Vogelstein et al.
	Art Unit	TBD
	Examiner Name	TBD
	Attorney Docket Number	001107.00794

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)	2009-11-11
Name/Print	Sarah A. Kagan	Registration Number	32141

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

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

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

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IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re the Application of:		Atty. Docket No.:	001107.00704
Bert VOGELSTEIN et al.			
Serial No.:	TBD	Group Art Unit:	TBD
Filed:	Herewith	Examiner:	TBD
For:	Digital Amplification		

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

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

Sir:

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

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

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

Respectfully submitted,
BANNER & WITCOFF, LTD.

Dated: November 11, 2009

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

Customer No. 22907

PATENT

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re Application of	)	Prior Group Art Unit: 1637
	)	
Bert VOGELSTEIN et al	)	Prior Examiner: S. Woolwine
	)	
Serial No. TBD	)	Confirmation No. TBD
	)	
Filed: Herewith	)	Atty. Dkt. No. 001107.00794
	)	
For: DIGITAL AMPLIFICATION	)	

SEQUENCE STATEMENT

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. No new matter is added.

Respectfully submitted,

Date: November 11, 2009

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

Banner & Witcoff, Ltd.
Customer No. 22907

SEQUENCE LISTING

<110> Vogelstein, Bert
Kinzler, Kenneth W.

<120> DIGITAL AMPLIFICATION

<130> 01107.00195

<140> 09/981,356
<141> 2001-10-12

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

<150> US 09/613,826
<151> 2000-07-11

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FIG. 1A

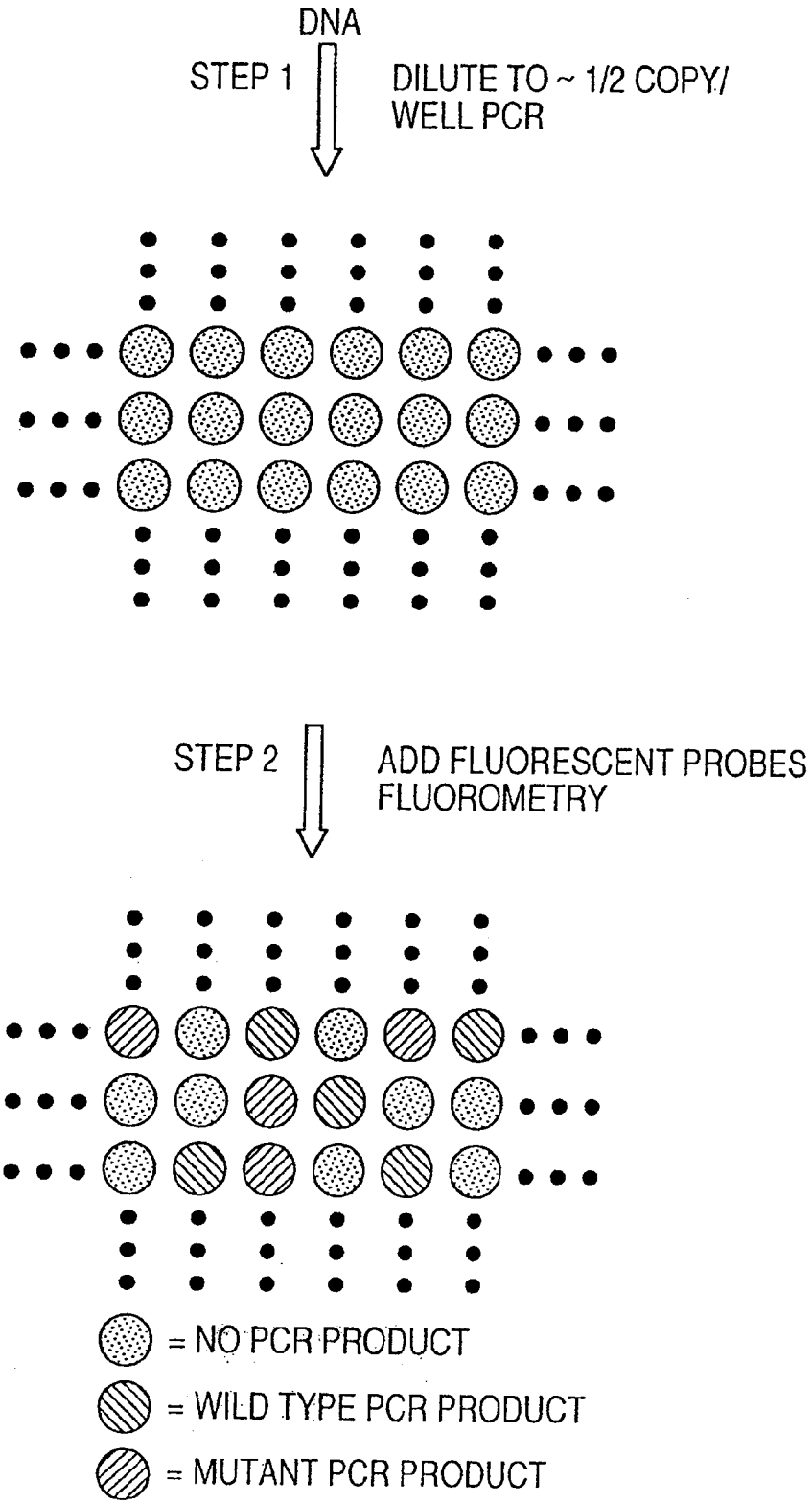
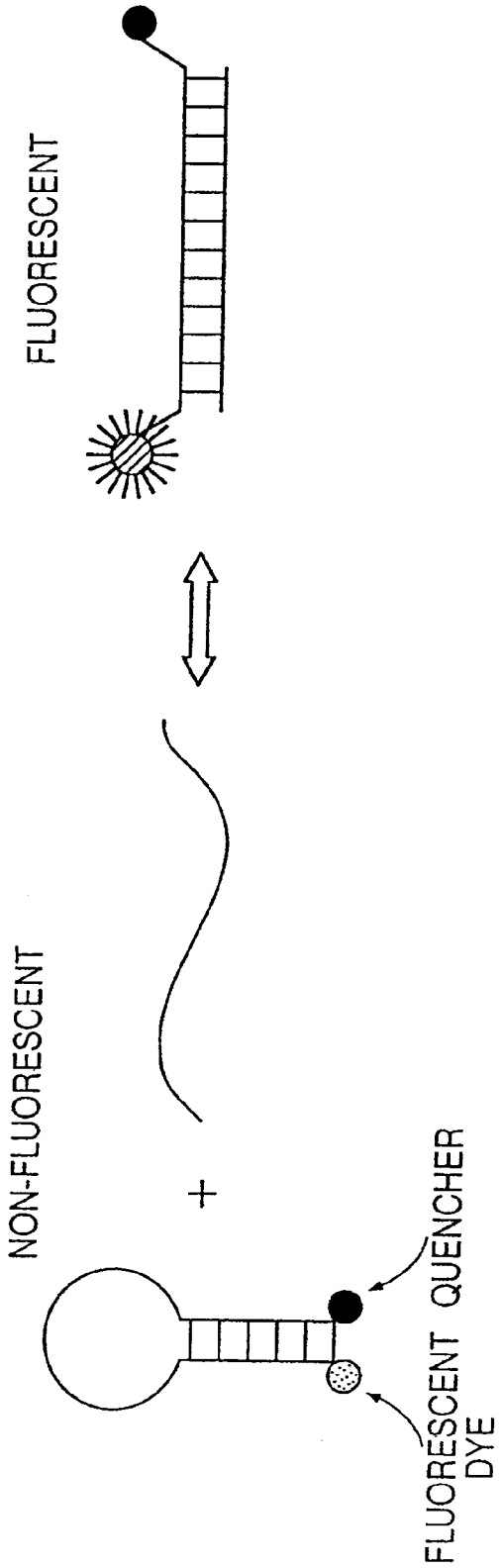

DNA
STEP 1
DILUTE TO ~ 1/2 COPY/ WELL PCR
STEP 2
ADD FLUORESCENT PROBES FLUOROMETRY
= NO PCR PRODUCT
= WILD TYPE PCR PRODUCT
= MUTANT PCR PRODUCT

FIG. 1B



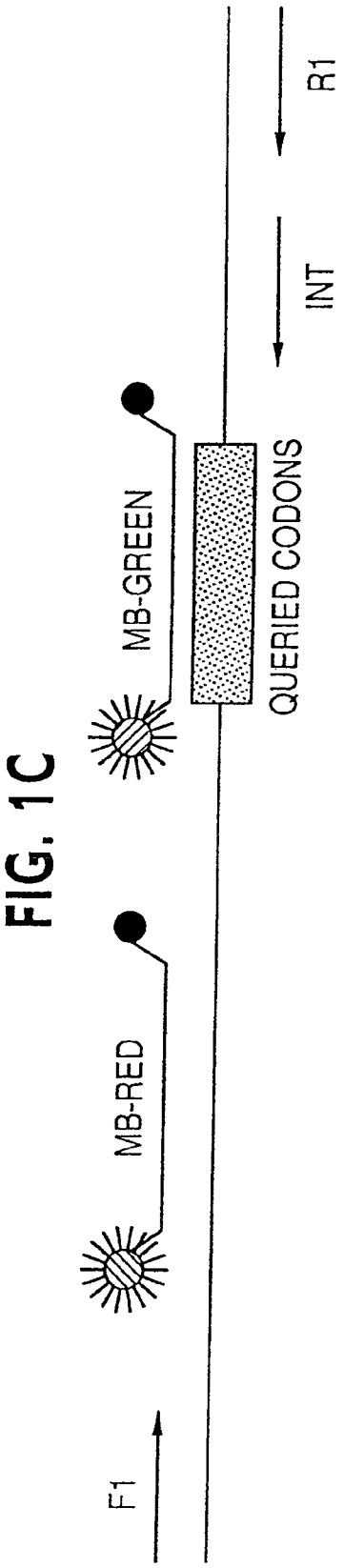
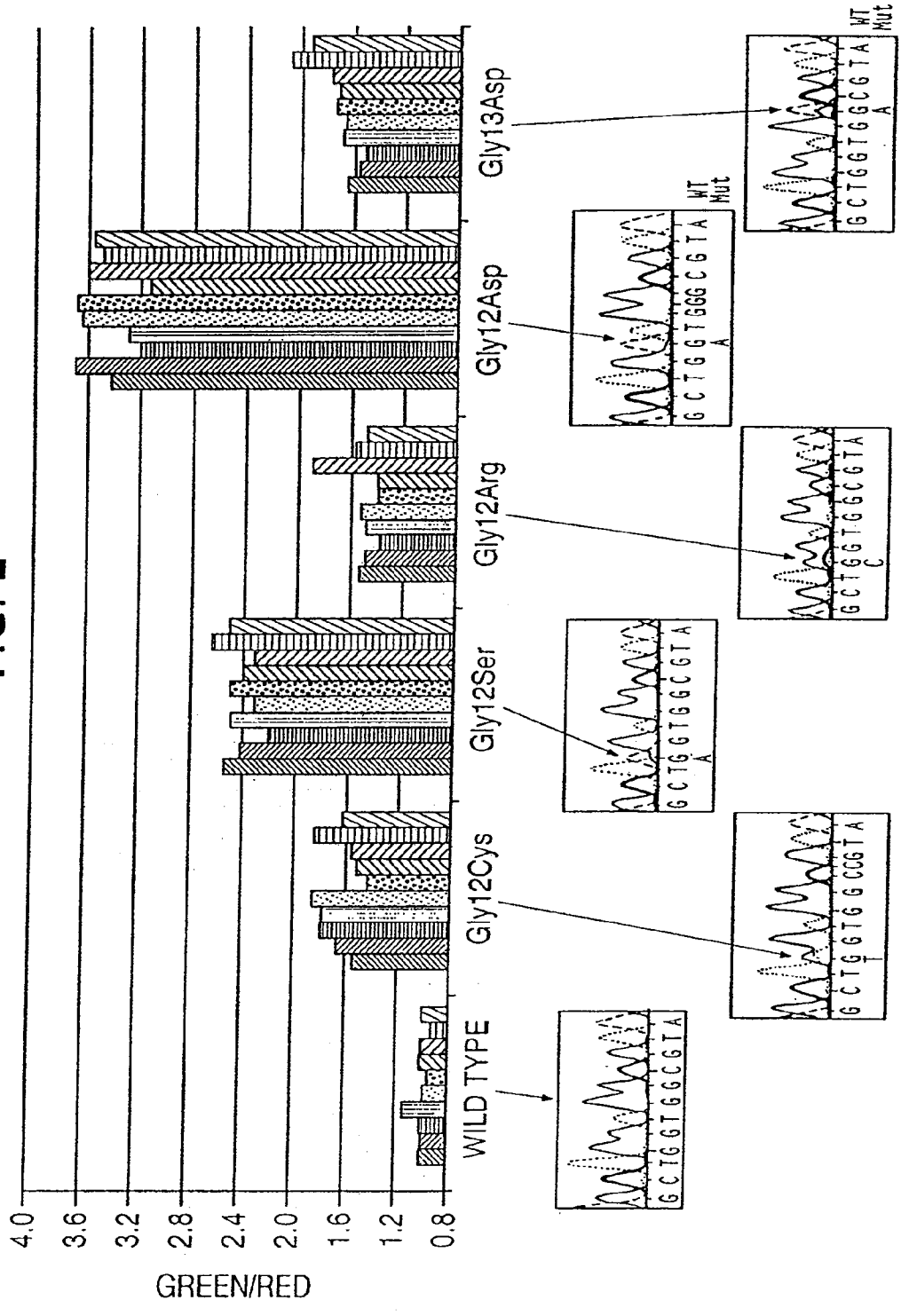


FIG. 1C

FIG. 2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3.6
3.2
2.8
2.4
2.0
1.6
1.2
0.8
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WILD TYPE
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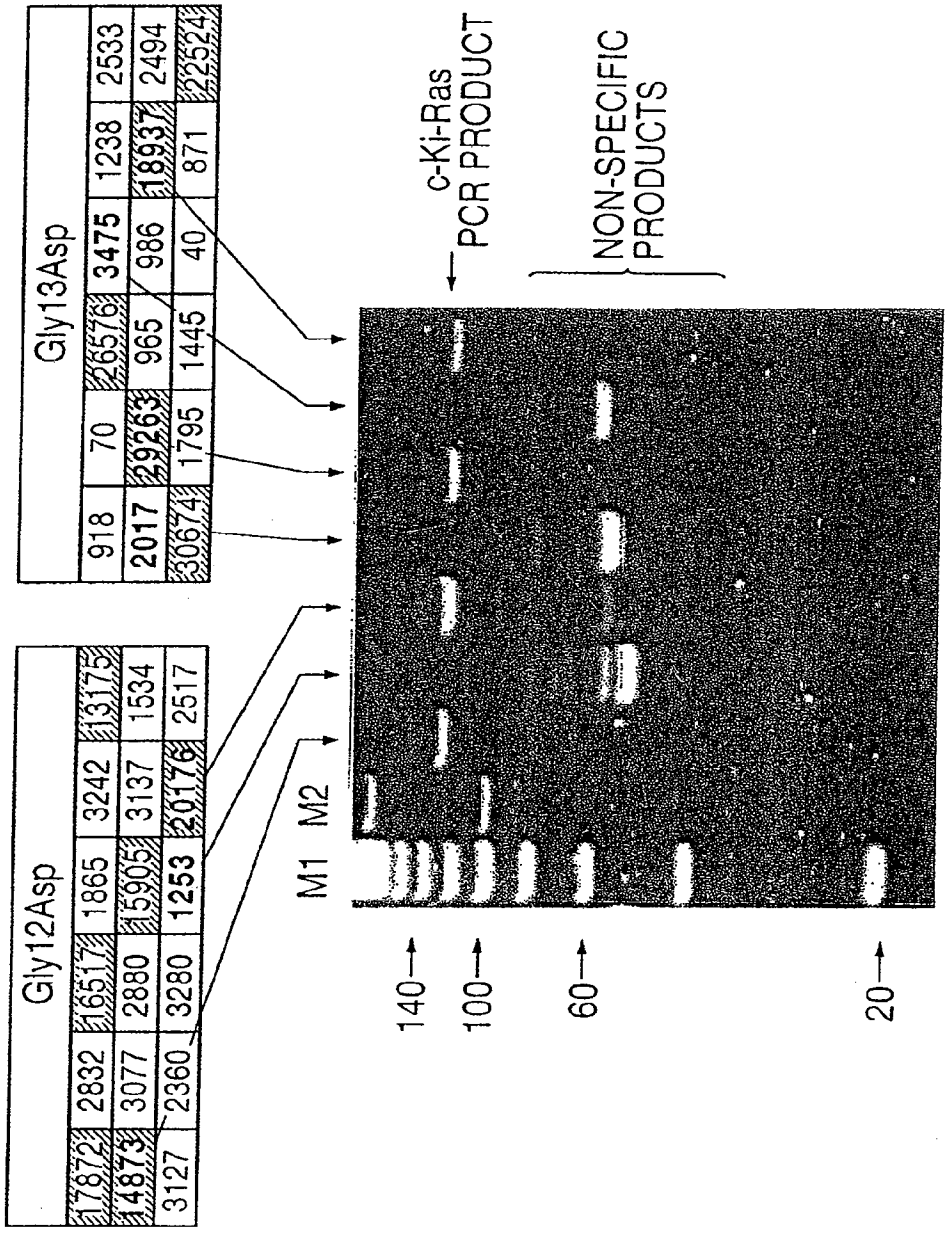
Gly12Arg
Gly12Asp
Gly13Asp
WT
Mut

FIG. 3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16517
1865
3242
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14873
3077
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3127
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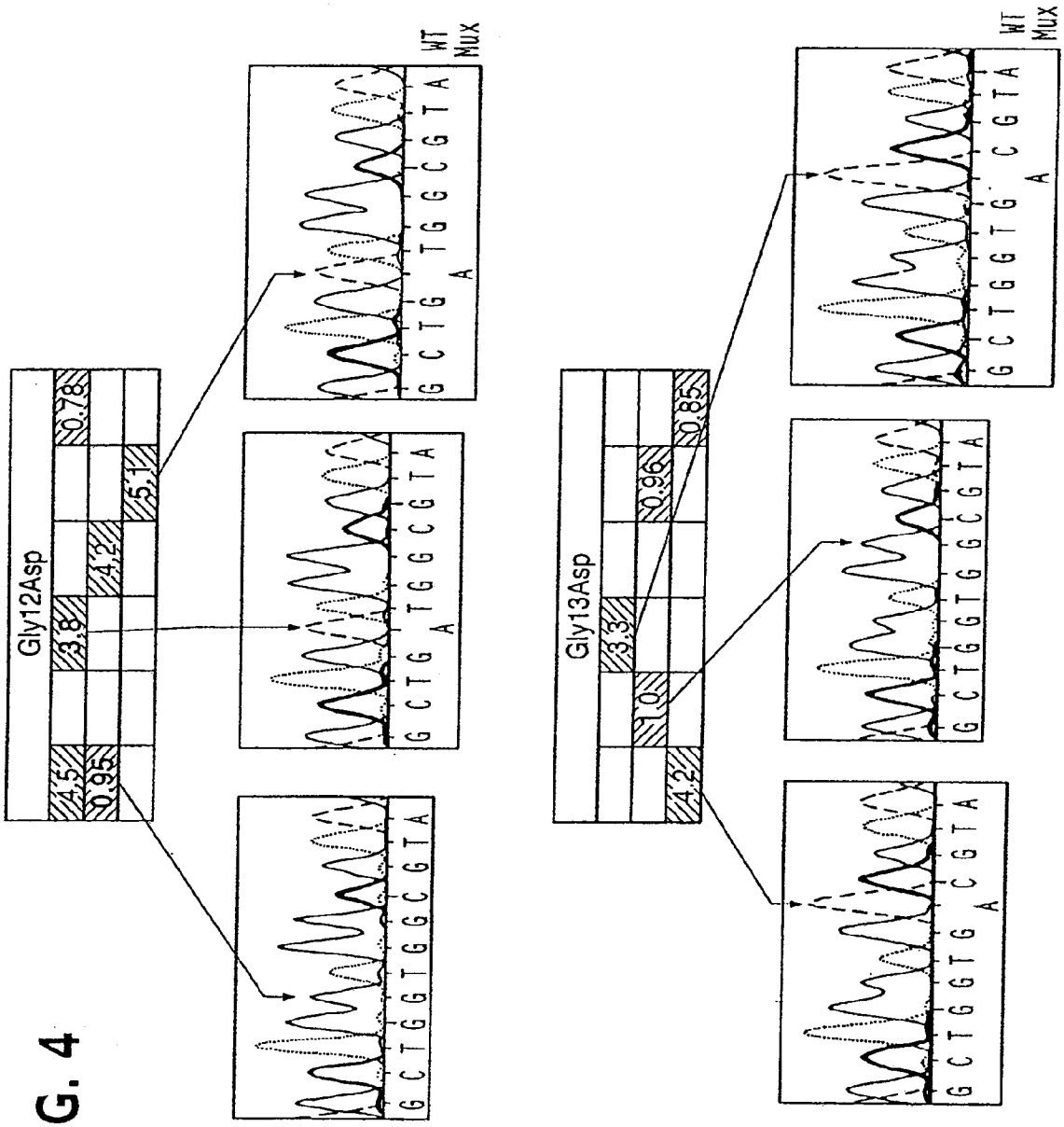
1253
20176
2517
Gly13Asp
918
70
26576
3475
1238
2533
2017
29263
965
986
18937
2494
30674
1795
1445
40
871
22524
M1
M2
c-Ki-Ras
PCR PRODUCT
NON-SPECIFIC
PRODUCTS
140
100
60
20

FIG. 4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0,95
3,8
4,2
5,1
0,78
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Mux
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G C T G A T G G C G T A
Gly13Asp
3,3
1,0
4,2
0,96
0,85
WT
Mux
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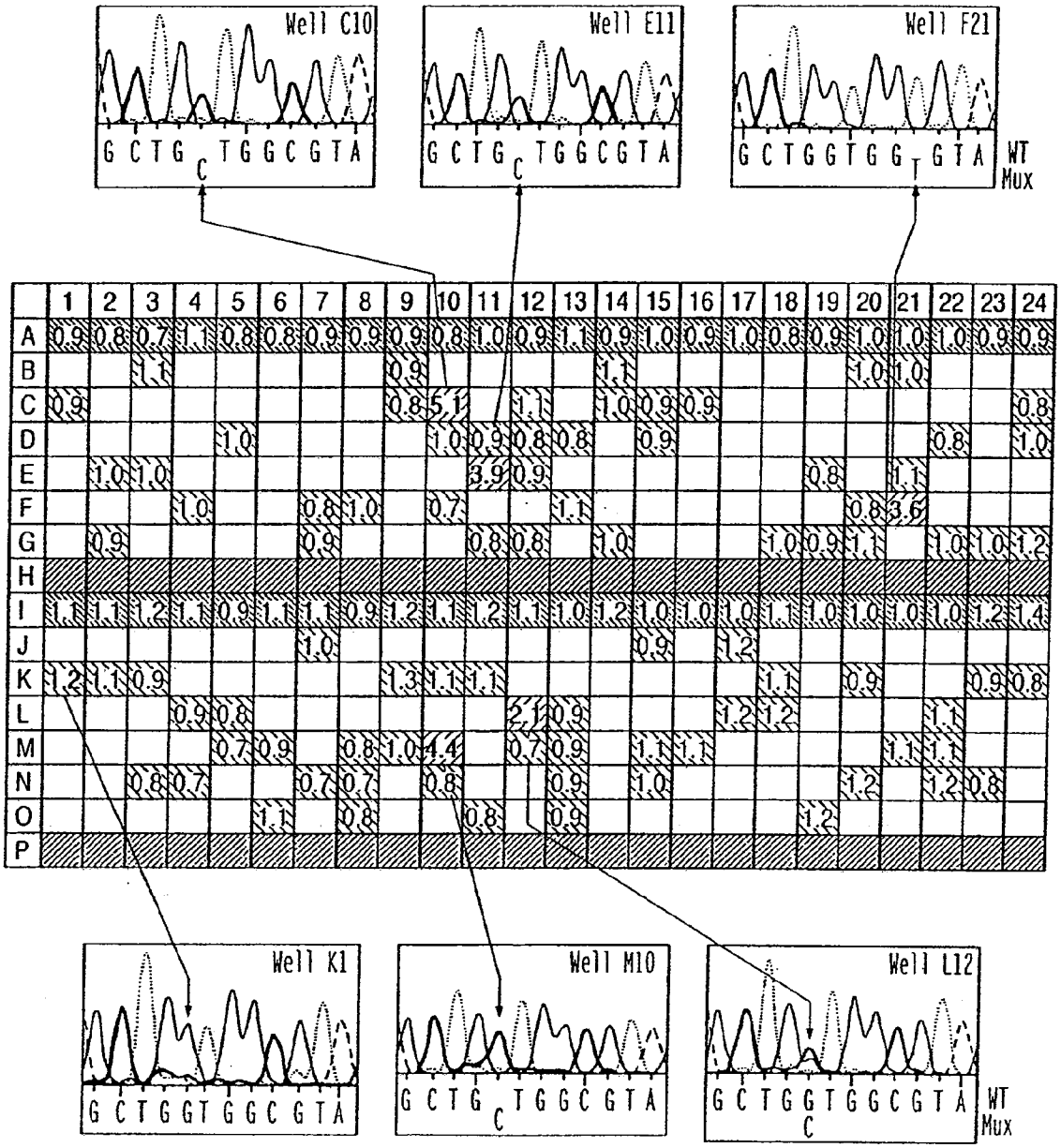
G C T G G T G A C G T A

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
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

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		

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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

DIGITAL AMPLIFICATION

This application is a division of U.S. Application Serial No. 11/709,742 filed February 23, 2007, which is a continuation of U.S. Application Serial Number 10/828,295 filed April 21, 2004, now abandoned, which is a division of U.S. Application Serial Number 09/981,356 filed October 12, 2001, now U.S. 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, now expired. 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 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 alleles mutated vs. one mutation in each of two alleles	first mutation	second mutation
Allelic Imbalance	Quantitative analysis with non-polymorphic markers	marker sequence	marker from another chromosome

The ultimate utility of Digital Amplification lies in its ability to convert the intrinsically exponential nature of PCR to a linear one. It should thereby

prove useful for experiments requiring the investigation of individual alleles, rare variants/mutations, or quantitative analysis of PCR products.

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

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

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

The number of different situations in which the digital amplification method will find application is large. Some of these are listed in Table 1. As

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

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

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

Photoluminescence also includes phosphorescence which denotes emission accompanying descent from an excited triplet or singlet state to a lower state of different multiplicity, *i.e.,* a quantum mechanically "forbidden" transition. Compared to "allowed" transitions, "forbidden" transitions are associated with relatively longer excited state lifetimes.

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

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

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

EXAMPLE 1

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

EXAMPLE 2

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

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

EXAMPLE 3

Oligonucleotides and DNA sequencing. Primer F1:
5'-CATGTTCTAATATAGTCACATTTTCA-3'; 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 Amplification assay was also consistent with the fraction of mutant alleles inferred from direct sequence analysis of genomic DNA from the two tumor lines (Fig. 2).

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

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

CLAIMS

1. A method for determining 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 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.

9. The method of claim 8 wherein the sample is from blood.

DIGITAL AMPLIFICATION

ABSTRACT

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

Electronic Patent Application Fee Transmittal

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Document Number	Document Description	File Name	File Size(Bytes)/ Message Digest	Multi Part /.zip	Pages (if appl.)
1	Transmittal Letter	1107transmittal794.pdf	147981 155c22ba248e705a937947b4a98a3023659edf4d	no	1
Warnings:					
Information:					
2	Application Data Sheet	1107ADS794.pdf	40285 9aab3960cc75d0750767d808f0f51ccce96c70f1	no	5
Warnings:					
Information:					
This is not an USPTO supplied ADS fillable form					
3	Information Disclosure Statement (IDS) Filed (SB/08)	1107IDSLTR794.pdf	67124 a4b3d25f6e2e9859d012aef4db26fee97d631ebc	no	1
Warnings:					
Information:					
This is not an USPTO supplied IDS fillable form					
4	Information Disclosure Statement (IDS) Filed (SB/08)	1107IDS794.pdf	39705 92d5e0a17f09f9eefcb0a95aa00996a595332707	no	7
Warnings:					
Information:					
This is not an USPTO supplied IDS fillable form					
5	Miscellaneous Incoming Letter	1107practitioners794.pdf	68732 8613be2e82b2f633a309727879590b6919da95a2	no	1
Warnings:					
Information:					
6	Sequence Listing	1107SEQSTMT794.pdf	57645 146ffd738fad86db334ec06c5372b72795cafc6a	no	1
Warnings:					
Information:					
7	Sequence Listing	1107SEQLISTING794.pdf	42212 db20e61d1cad3993ed576de878b7966e41d23324	no	3
Warnings:					
Information:					

8	Drawings-only black and white line drawings	1107drawings794.pdf	353535 69f3d0818b17aa66c9fec5e091fe1e9baeeabf8e	no	7
Warnings:					
Information:					
9	Oath or Declaration filed	1107declaration794.pdf	112006 68b6159cf735266d77656bec8bcbce2c1ed60a50	no	2
Warnings:					
Information:					
10	Specification	1107specification794.pdf	207102 5940eeb60987d6fc909b4c080d343415ebc955ab	no	25
Warnings:					
Information:					
11	Fee Worksheet (PTO-875)	fee-info.pdf	32334 c4886d296fab0df3bf9aac8e4488122c6ac3ee37	no	2
Warnings:					
Information:					
		Total Files Size (in bytes):	1168661		

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/05 (02-07)
Approved for use through 02/28/2007. 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 CFR 1.53(b))

Attorney Docket No.	001107.00794
First Inventor	Bert VOGELSTEIN et al.
Title	Digital Amplification
Express Mail Label No.	

APPLICATION ELEMENTS

See MPEP chapter 600 concerning utility patent application contents.

1. ☐ **Fee Transmittal Form** (e.g., PTO/SB/17) *(Submit an original and a duplicate for fee processing)*
2. ☐ **Applicant claims small entity status.** See 37 CFR 1.27.
3. ☒ **Specification** [*Total Pages* 26] Both the claims and abstract must start on a new page *(For information on the preferred arrangement, see MPEP 608.01(a))*
4. ☒ **Drawing(s)** (*35 U.S.C. 113*) [*Total Sheets* 7]
5. **Oath or Declaration** [*Total Sheets* 2]
   a. ☐ Newly executed (original or copy)
   b. ☒ A copy from a prior application (37 CFR 1.63(d)) *(for continuation/divisional with Box 18 completed)*
      i. ☐ DELETION OF INVENTOR(S) Signed statement attached deleting inventor(s) name in the prior application, see 37 CFR 1.63(d)(2) and 1.33(b).
6. ☒ **Application Data Sheet.** See 37 CFR 1.76
7. ☐ **CD-ROM or CD-R** in duplicate, large table or Computer Program *(Appendix)*
   ☐ Landscape Table on CD
8. **Nucleotide and/or Amino Acid Sequence Submission** *(if applicable, items a. – c. are required)*
   a. ☒ Computer Readable Form (CRF)
   b. Specification Sequence Listing on:
      i. ☐ CD-ROM or CD-R (2 copies); or
      ii. ☒ Paper
   c. ☒ Statements verifying identity of above copies

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

ACCOMPANYING APPLICATION PARTS

9. ☐ **Assignment Papers** (cover sheet & document(s))
   Name of Assignee _____
10. ☐ **37 CFR 3.73(b) Statement** *(when there is an assignee)* ☐ **Power of Attorney**
11. ☐ **English Translation Document** *(if applicable)*
12. ☒ **Information Disclosure Statement** (PTO/SB/08 or PTO-1449)
    ☐ Copies of foreign patient documents, publications, & other information
13. ☐ **Preliminary Amendment**
14. ☐ **Return Receipt Postcard** (MPEP 503) (*Should be specifically itemized*)
15. ☐ **Certified Copy of Priority Document(s)** *(if foreign priority is claimed)*
16. ☐ **Nonpublication Request** under 35 U.S.C. 122(b)(2)(B)(i). Applicant must attach form PTO/SB/35 or equivalent.
17. ☐ Other: _____

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.: 11/709,742

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

19. CORRESPONDENCE ADDRESS

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

Name					
Address					
City		State		Zip Code	
Country		Telephone		Email	

Signature	/Sarah A. Kagan/	Date	November 11 , 2009	
Name (Print/Type)	Sarah A. Kagan		Registration No. (Attorney/Agent)	32,141

PTO/SB/14 (07-07)
Approved for use through 06/30/2010. OMB 0651-0032
U.S. Patent and Trademark Office; U.S. DEPARTMENT OF COMMERCE

Application Data Sheet 37 CFR 1.76	Attorney Docket Number	001107.00794
	Application Number	
Title of Invention	Digital Amplification	

The application data sheet is part of the provisional or nonprovisional application for which it is being submitted. The following form contains the bibliographic data arranged in a format specified by the United States Patent and Trademark Office as outlined in 37 CFR 1.76.
This document may be completed electronically and submitted to the Office in electronic format using the Electronic Filing System (EFS) or the document may be printed and included in a paper filed application.

Secrecy Order 37 CFR 5.2

☐ Portions or all of the application associated with this Application Data Sheet may fall under a Secrecy Order pursuant to 37 CFR 5.2 (Paper filers only. Applications that fall under Secrecy Order may not be filed electronically.)

Applicant Information:

Applicant 1

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

Prefix	Given Name	Middle Name	Family Name	Suffix
	Bert		VOGELSTEIN	

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

City	Baltimore	State/Province	MD	Country of Residence	US

Citizenship under 37 CFR 1.41(b) US

Mailing Address of Applicant:

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

Applicant 2

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

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

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

City	Baltimore	State/Province	MD	Country of Residence	US

Citizenship under 37 CFR 1.41(b) US

Mailing Address of Applicant:

Address 1	1403 Halkirk Way		
Address 2			
City	Baltimore	State/Province	MD
Postal Code	21015	Country	US

All Inventors Must Be Listed - Additional Inventor Information blocks may be generated within this form by selecting the **Add** button. Add

Correspondence Information:

Enter either Customer Number or complete the Correspondence Information section below.
For further information see 37 CFR 1.33(a).

☐ **An Address is being provided for the correspondence Information of this application.**

PTO/SB/14 (07-07)
Approved for use through 06/30/2010. 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 contains a valid OMB control number.

Application Data Sheet 37 CFR 1.76	Attorney Docket Number	001107.00794
	Application Number	
Title of Invention	Digital Amplification	

Customer Number	22907		
Email Address		Add Email	Remove Email

Application Information:

Title of the Invention	Digital Amplification		
Attorney Docket Number	001107.00794	Small Entity Status Claimed	☐
Application Type	Nonprovisional		
Subject Matter	Utility		
Suggested Class (if any)		Sub Class (if any)	
Suggested Technology Center (if any)			
Total Number of Drawing Sheets (if any)		Suggested Figure for Publication (if any)	

Publication Information:

☐ Request Early Publication (Fee required at time of Request 37 CFR 1.219)

☐ **Request Not to Publish.** I hereby request that the attached application not be published under 35 U.S. C. 122(b) and certify that the invention disclosed in the attached application **has not and will not** be the subject of an application filed in another country, or under a multilateral international agreement, that requires publication at eighteen months after filing.

Representative Information:

Representative information should be provided for all practitioners having a power of attorney in the application. Providing this information in the Application Data Sheet does not constitute a power of attorney in the application (see 37 CFR 1.32).
Enter either Customer Number or complete the Representative Name section below. If both sections are completed the Customer Number will be used for the Representative Information during processing.

Please Select One:	◉ Customer Number	○ US Patent Practitioner	○ Limited Recognition (37 CFR 11.9)
Customer Number	22907		

Domestic Benefit/National Stage Information:

This section allows for the applicant to either claim benefit under 35 U.S.C. 119(e), 120, 121, or 365(c) or indicate National Stage entry from a PCT application. Providing this information in the application data sheet constitutes the specific reference required by 35 U.S.C. 119(e) or 120, and 37 CFR 1.78(a)(2) or CFR 1.78(a)(4), and need not otherwise be made part of the specification.

Prior Application Status	Pending		Remove
Application Number	Continuity Type	Prior Application Number	Filing Date (YYYY-MM-DD)
	Division of	11709742	2007-02-23
Prior Application Status	Abandoned		Remove
Application Number	Continuity Type	Prior Application Number	Filing Date (YYYY-MM-DD)
11709742	Continuation of	10828295	2004-04-21

PTO/SB/14 (07-07)
Approved for use through 06/30/2010. OMB 0651-0032
U.S. Patent and Trademark Office; U.S. DEPARTMENT OF COMMERCE

Application Data Sheet 37 CFR 1.76	Attorney Docket Number	001107.00794
	Application Number	
Title of Invention	Digital Amplification	

Prior Application Status	Patented				Remove
Application Number	Continuity Type	Prior Application Number	Filing Date (YYYY-MM-DD)	Patent Number	Issue Date (YYYY-MM-DD)
10828295	Division of	09981356	2001-10-12	6753147	2004-06-22
Prior Application Status	Patented				Remove
Application Number	Continuity Type	Prior Application Number	Filing Date (YYYY-MM-DD)	Patent Number	Issue Date (YYYY-MM-DD)
09981356	Continuation of	09613826	2000-07-11	6440706	2002-08-27
Prior Application Status	Expired				Remove
Application Number	Continuity Type	Prior Application Number	Filing Date (YYYY-MM-DD)		
09613826	non provisional of	60146792	1999-08-02		

Additional Domestic Benefit/National Stage Data may be generated within this form by selecting the **Add** button.

Foreign Priority Information:

This section allows for the applicant to claim benefit of foreign priority and to identify any prior foreign application for which priority is not claimed. Providing this information in the application data sheet constitutes the claim for priority as required by 35 U.S.C. 119(b) and 37 CFR 1.55(a).

Remove

Application Number	Country	Parent Filing Date (YYYY-MM-DD)	Priority Claimed
			◉ Yes ◉ No

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

Assignee Information:

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

Assignee 1

If the Assignee is an Organization check here. ☒

Organization Name	The Johns Hopkins University		
Mailing Address Information:			
Address 1	3400 N. Charles Street		
Address 2			
City	Baltimore	**State/Province**	MD
Country	US	Postal Code	21218
Phone Number		Fax Number	
Email Address			

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

Signature:

A signature of the applicant or representative is required in accordance with 37 CFR 1.33 and 10.18. Please see 37 CFR 1.4(d) for the form of the signature.

PTO/SB/14 (07-07)
Approved for use through 06/30/2010. OMB 0651-0032
U.S. Patent and Trademark Office; U.S. DEPARTMENT OF COMMERCE

Application Data Sheet 37 CFR 1.76	Attorney Docket Number	001107.00794
	Application Number	
Title of Invention	Digital Amplification	

Signature	/Sarah A. Kagan/			Date (YYYY-MM-DD)	2009-11-11
First Name	Sarah A.	Last Name	Kagan	Registration Number	32141

This collection of information is required by 37 CFR 1.76. The information is required to obtain or retain a benefit by the public which is to file (and by the USPTO to process) an application. Confidentiality is governed by 35 U.S.C. 122 and 37 CFR 1.14. This collection is estimated to take 23 minutes to complete, including gathering, preparing, and submitting the completed application data sheet form to the USPTO. Time will vary depending upon the individual case. Any comments on the amount of time you require to complete this form and/or suggestions for reducing this burden, should be sent to the Chief Information Officer, U.S. Patent and Trademark Office, U.S. Department of Commerce, P.O. Box 1450, Alexandria, VA 22313-1450. DO NOT SEND FEES OR COMPLETED FORMS TO THIS ADDRESS. **SEND TO: Commissioner for Patents, P.O. Box 1450, Alexandria, VA 22313-1450.**

Privacy Act Statement

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

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

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

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

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

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

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

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

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

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

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

EXHIBIT 4

ISSUE CLASSIFICATION	Class	Subclass
	435	91.2

PATENT NUMBER
6440705
6440705

U.S. UTILITY Patent Application

O.I.P.E. SCANNED	PATENT DATE
KW GW Q.A. CL	AUG 27 2002

APPLICATION NO.	CONT/PRIOR	CLASS	SUBCLASS	ART UNIT	EXAMINER
09/613826	D	435	91.2	~~1638~~ 1657	Sircus 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 Sheets Drwg.	Figs. Drwg.	Print Fig.
7	7	None

CLAIMS ALLOWED Total Claims	Print Claim for O.G.
64	1

NOTICE OF ALLOWANCE MAILED: March 26, 2002

ISSUE FEE (W)

Amount Due	Date Paid
$1640.00	5-21-02

_____ (Assistant Examiner) _____ (Date)

Jeffrey Siew 3/19/02
(Primary Examiner) (Date)

MONICA R. GRAVES PATENT ANALYST 3/22/02
(Legal Instruments Examiner) (Date)

ISSUE BATCH NUMBER: 9893

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Form PTO-436A
(Rev. 6/99)

FILED WITH: ☐ DISK (CRF) ☐ FICHE ☐ CD-ROM
(Attached in pocket on right inside flap)

ISSUE FEE IN FILE

[redacted]

Formal Drawings (___ sht(s)) ent (FACE)

PATENT APPLICATION

09613826

JC869 U.S. PTO
09/613826
07/11/00

Briefed in HCO

INITIALS

CONTENTS

		Date Received (Incl. C. of M.) or Date Mailed		Date Received (Incl. C. of M.) or Date Mailed
1.	Application ____ papers.		42.	
2.	Ltr re Filing claim, Dec missing	10-31-00	43.	
3.	PE C	03-22-01	44.	
4.	IDS	12-15-00	45.	
5.	IDS	3-20-01	46.	
6.	Rej (3rd)	4-17-01 4/19	47.	
7.	IDS	7/12/01	48.	
8.	Amdt A Req Extn Advit	7/12/01	49.	
9.	Supple. Disclosure Statement	07/26/01	50.	
10.	Final Rej (3)	9/20/01 9/20	51.	
11.	RSL	2/24/01	52.	
12.	RSL	9-28-01	53.	
13.	Amdt B NIE	12-06-01	54.	
14.	Advisory Actn	12-13-01 12/12	55.	
15.	IDS	1/3/02	56.	
16.	Ext of time (2)	2/20/02	57.	
17.	Amdt C (NE)	2/20/02	58.	
18.	Interview Summary	3/4/02	59.	
19.	RAW SEQ LISTING	3/1/02	60.	
20.	Interview Summary	3/19/02	61.	
21.	Notice of Allowability	3/26/02	62.	
22.	Suppl Notice of Allowability	3/26/02	63.	
23.	Formal Drawing	5/21/02	64.	
24.	Ltr Re Drawings	7/15/02	65.	
7-23-02 25.	Formal Drawings (7 sht's) rec'd	7-8-02	66.	1
26.			67.	1
27.			68.	
28.			69.	
29.			70.	
30.			71.	
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40.			81.	
41.			82.	

(LEFT OUTSIDE)

SEARCHED

Class	Sub.	Date	Exmr.
435	6	4/7/01	JS
	7.1		
	91.1		
	91.2		
536	22.1		
	23.1		
	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			
O.I.P.E. CLASSIFIER			
FORMALITY REVIEW		551	8-29-01
RESPONSE FORMALITY REVIEW		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
	1	✓
	2	✓
	3	✓
	4	✓
	5	✓
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	49	✓
	50	✓

Claim Final	Claim Original	Date
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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

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 ** 08/29/2000

** SMALL ENTITY **

Foreign Priority claimed	STATE OR COUNTRY	SHEETS DRAWING	TOTAL CLAIMS	INDEPENDENT CLAIMS
☐ yes ☒ no	MD	7	64	5

35 USC 119 (a-d) conditions met ☐ yes ☒ no ☐ Met after Allowance

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TITLE

Digital amplification

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☐ yes ☐ no ☐ Met after Allowance

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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

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).

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CLAIMS

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

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.

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Fig. 1A

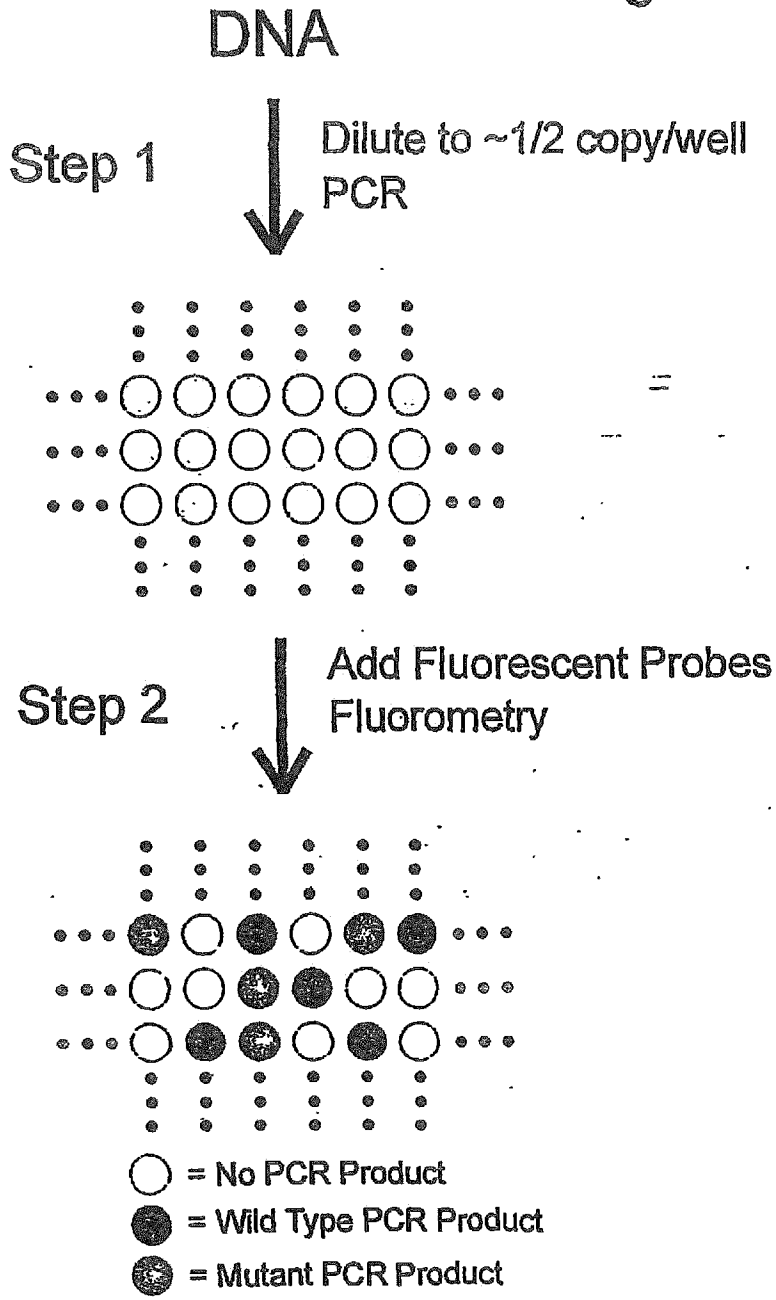

Fig. 1B

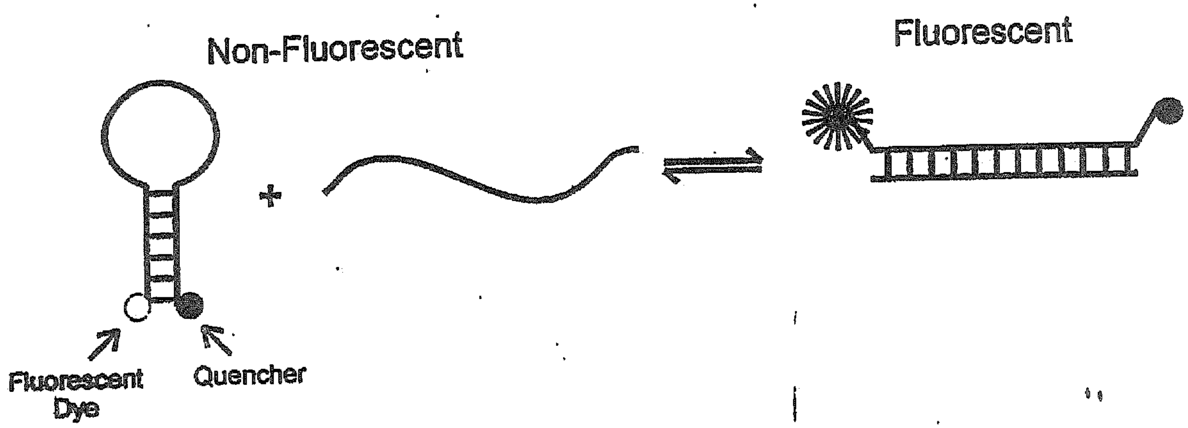

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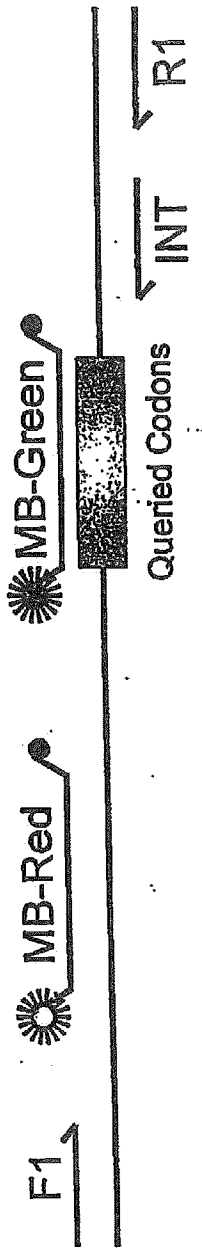
Fig. 1C



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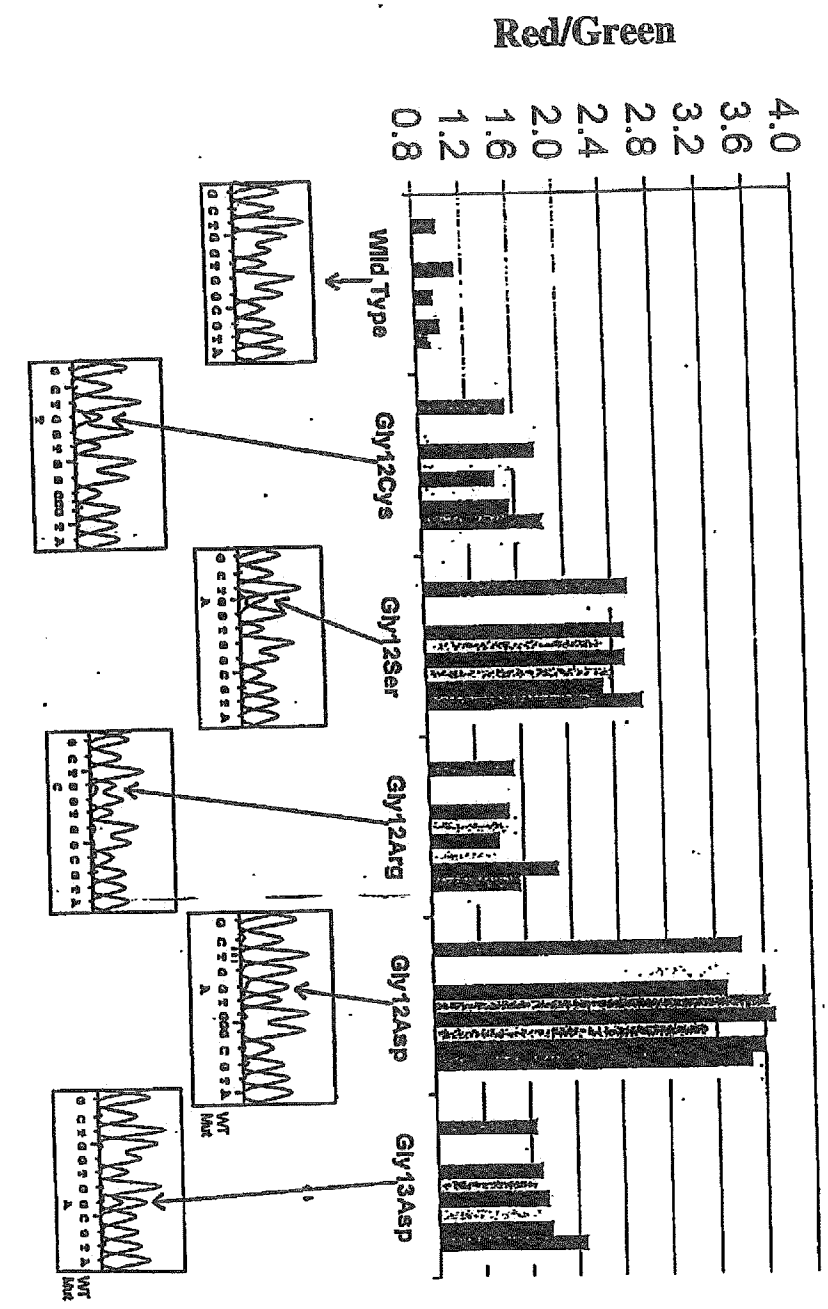

Fig. 2

Fig. 3

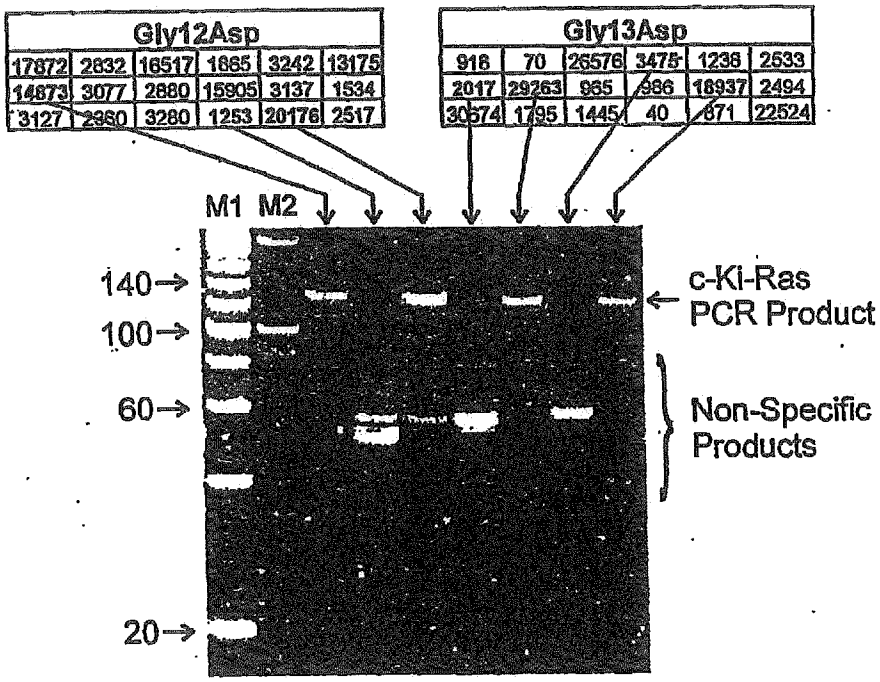

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Fig. 4

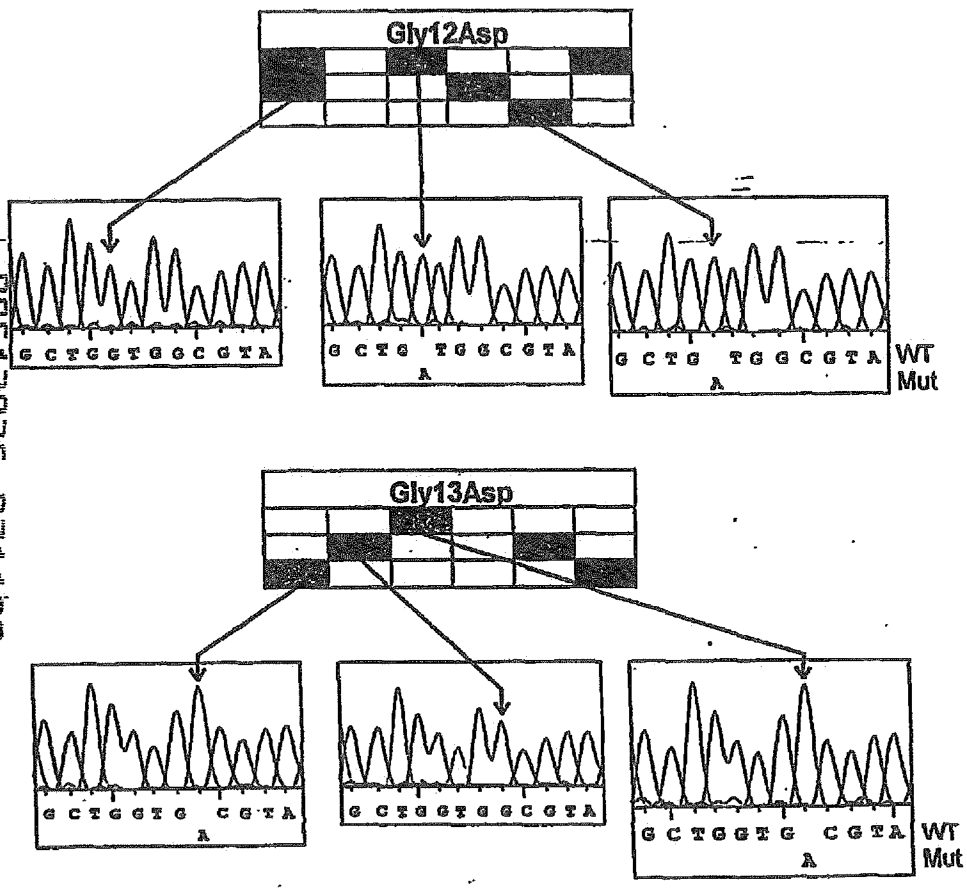

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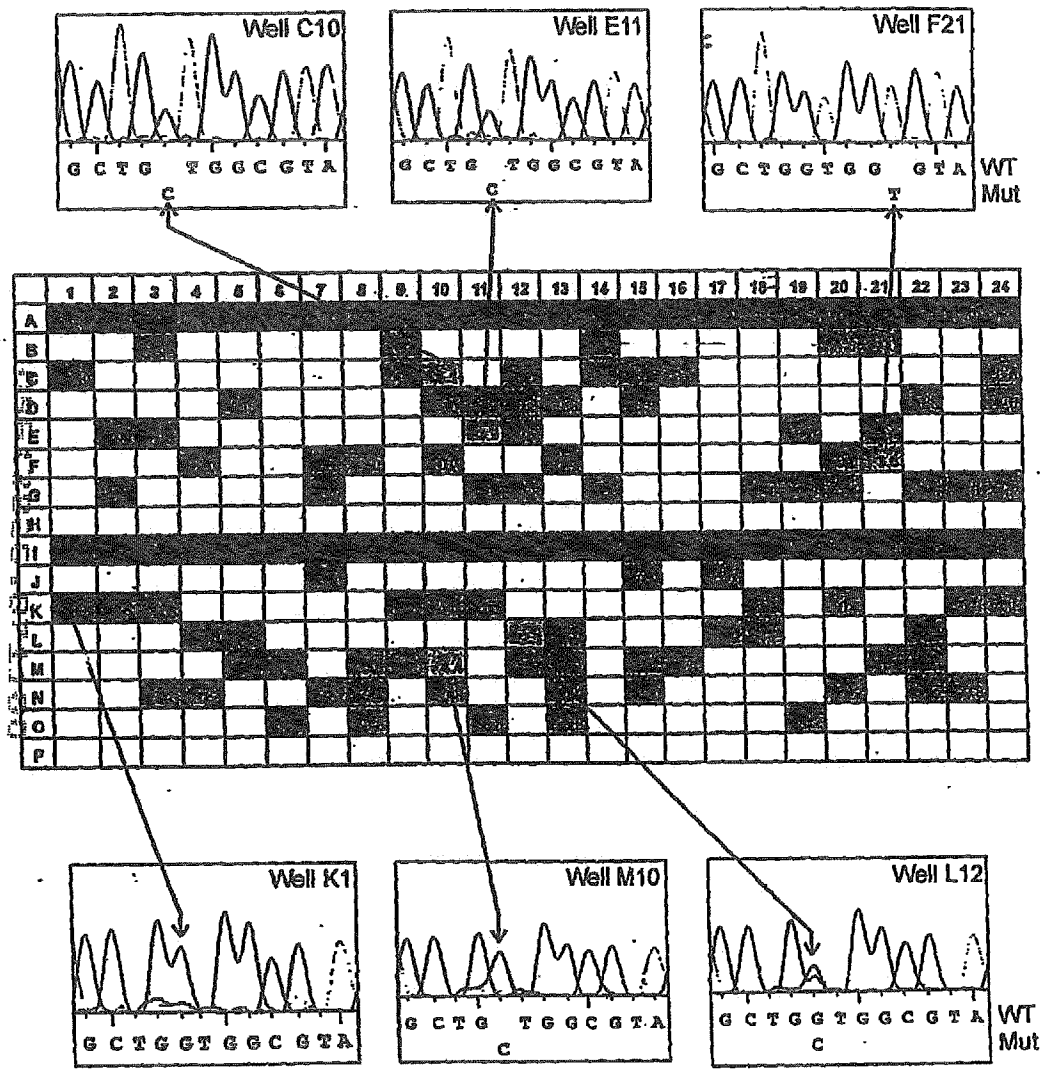
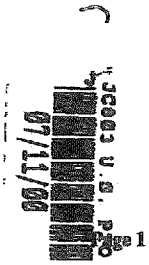
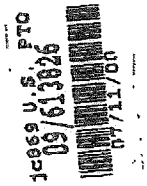
Fig. 5







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

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

FORMALITIES LETTER

OC000000005521419

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1001 G Street N W
Washington, DC 20001-4597

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. t office address and citizenship are as stated below next to our names;

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

- ☐ is atta.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	PRATT, 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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355.00 CH
80.00 CH
356.00 CH
12/19/2000 SMUMS
01 FC:201
02 FC:202
03 FC:203
04 FC:205

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

#115

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

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.*

Serial No.: 09/613,826

Filed: July 11, 2000

Group Art No. 1632

Examiner: TBA

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

Sheet 1 of 1

#5

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

RECEIVED
MAR 08 2001
TECH CENTER 1600/2900

OIPE
MAR 07 2001
PATENT & TRADEMARK OFFICE

U.S. PATENT DOCUMENTS

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

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

	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, September 29, 1988 pp 414-417
	Ramon PARSONS, *et al.*, "Mismatch Repair Deficiency in Phenotypically Normal Human Cells", Science, Vol. 268, May 5, 1995 pp 738-740
	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, 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, July 1998 pp 225-232
	~~W. NAVIDI, *et al.*, "Using PCR in Preimplantation Genetic Disease Diagnosis", Human Reproduction, Vol. 6, 1991~~
	~~Hongiua 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 Slew	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

Sheet 1 of 2

PTO-1449 (Modified) U.S. DEPARTMENT OF COMMERCE PATENT AND TRADEMARK OFFICE INFORMATION DISCLOSURE STATEMENT BY APPLICANT	ATTY. DOCKET NO. 01107.0003	SERIAL NUMBER ~~09/613,826~~ 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 tumour progression" Nature Vol. 355, pages 846-847 1992
JS	Navidi et al. "Using PCR in preimplantation genetic disease diagnosis" Human reproductionVol. 6, No. 6, pages 836-849 1991

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.

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

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

MARKED UP VERSION TO SHOW CHANGES MADE

Replacement paragraph beginning on page 4, line 5.

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

Replacement paragraph beginning on page 14, line 29.

Oligonucleotides and DNA sequencing. Primer F1: 5'-CATGTTCTAATATAGTCACATTTTCA-3' (SEQ ID NO: 1); Primer R1: 5'-TCTGAATTAGCTGTATCGTCAAGG-3' (SEQ ID NO: 2); Primer INT: 5'-TAGCTGTATCGTCAAGGCAC-3' (SEQ ID NO: 3); MB-RED:

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:

For Rules Interpretation, call (703) 308-4216
For CRF Submission Help, call (703) 308-4212
Patentin Software Program Support
Technical Assistance.....703-287-0200
To Purchase Patentin Software.....703-306-2600

PLEASE RETURN A COPY OF THIS NOTICE WITH YOUR REPLY

A

☒ 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/bf
Inventor VOGELSTEIN, et al. Client Jones Hansen
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
☐ Small Entity Declaration
☒ 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
☐ Response to Missing Parts/Rqmts. dtd _____
☐ Request for Expedited Foreign Filing License
☐ Request for Corrected : ☐ Filing Rec ☐ Assignment
☐ Response to Restriction/Election Requirement

☐ Sequence Listing : ☐ Diskette ☐ Paper
☐ Amendment ☐ Response : OA dtd _____
☐ CPA ☐ RCE ☐ w/Ext of Time : OA dtd _____
☐ Petition for Extension of Time until _____
☐ Request for Approval of Drawing Changes
☐ Notice of Appeal & Fee
☐ Brief : ☐ Appeal & Fee ☐ Reply
☐ Request for Oral Hearing
☐ Issue Fee ☐ Adv Patent Copies (#ordered ____)
Notice of Allowance dtd _____
☐ Amendment under 37 CFR 1.312
☐ Request for Certificate of Correction
☐ Check #_____ for $ _____
☐ Check #_____ for $ _____
☐ _____
☐ _____
☐ _____
☐ _____

B&W Rec 10/00

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

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 ~~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 Varient 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
	Hongua 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 # 0116.5003i Atty/Sec SAK/amc Date 12/15/00

Inventor [illegible] Client IBM

Title Digital Authentication

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# _____

☐ 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

☐ CPA Request ☐ w/Ext of Time : OA dtd _____

☐ Notice of Appeal & Fee

☐ Brief : ☐ Appeal & Fee ☐ Reply

Patent/Design ☐ PATENT ☐ DESIGN

☐ HAND CARRY Group/Section _____ Bldg _____ Rm ____

Serial No. 09/613,526 B&W # 0116.5003i Atty/Sec SAK/amc Date 12/15/00

Inventor [illegible] Client IBM

Title Digital Authentication

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# _____

☐ 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

☐ CPA Request ☐ w/Ext of Time : OA dtd _____

☐ Notice of Appeal & Fee

☐ Brief : ☐ Appeal & Fee ☐ Reply

☐ Request for Oral Hearing

☐ Issue Fee : Allowance dtd _____

☐ Advance Patent Copies : # ordered _____

☐ Check # _____ for $ _____

☐ Check # _____ for $ _____

☐ _____

☐ _____

☐ _____

☐ _____

☒ Declaration/PoA : ☒ Executed ☐ Unexecuted

☐ Drawings : ☐ Formal ☐ Informal # of distinct sheets _____ Figs. _____

☐ Assignment w/PTO Cover Sheet

☐ Small Entity Declaration

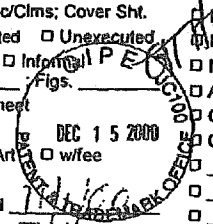
☒ IDS w/PTO 1449 ☐ Prior Art ☐ w/fee

☐ Preliminary Amendment

☒ Response : Missing Parts dtd 1/16/01

☐ Request for Expedited Foreign Filing License

☐ Request for Corrected Filing Receipt



12

B&W Rev 4/99

Box Seq 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)

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
- ☐ 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)*:
  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: ____

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.

406795-1

4

SEQUENCE LISTING

<110> Vogelstein, Bert
Kinzler, Kenneth W.

<120> DIGITAL AMPLIFICATION

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<140> 09/613,826
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PATENT

#9
MB
07/23/01

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

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

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
WASHINGTON, DC 20001
202-508-9100

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)

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
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- ☐ Response to Missing Parts/ Incomplete Application
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- ☐ Assignment Papers *(for an Application)*
- ☐ Drawing(s)
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- ☐ 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	D 0110/0003J

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

EXAMINER	
STEW, T	
ART UNIT	**PAPER NUMBER**
1656	10

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
TIME: 11:12:16

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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 DHW 10-3-01

1656

OIPE

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Alpha or Numeric: Numeric
Application Class:
Application File Date: 07-11-2000
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Software Application: PatentIn
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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'.

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PTO/SB/21 (08-00)

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- ☐ Fee Transmittal Form
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Firm or Individual name	Michelle Holmes-Son, Registration No. 47,660
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