EXHIBIT 4

ISSUE CLASSIFICATION	Class	Subclass
	435	91.2

PATENT NUMBER
6440705
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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	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								

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☐ The term of this patent subsequent to _____ (date) has been disclaimed.

☐ The term of this patent shall not extend beyond the expiration date of U.S Patent. No. _____

☐ The terminal ___ months of this patent have been disclaimed.

DRAWINGS			CLAIMS ALLOWED	
Sheets Drwg.	Figs. Drwg.	Print Fig.	Total Claims	Print Claim for O.G.
7	7	None	64	1

NOTICE OF ALLOWANCE MAILED

_____ (Assistant Examiner) _____ (Date)

March 26, 2002

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

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

ISSUE BATCH NUMBER
9893

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ISSUE FEE IN FILE

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Formal Drawings ( ) sht(s) rec'd (FACE)

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PATENT APPLICATION

09613826

JC869 U.S. PTO
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Briefed in HCO

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updated		9/20/01	JS
updated		3/19/02	JS

INTERFERENCE SEARCHED

Class	Sub.	Date	Exmr.
435	6	3/19/02	JS
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	24.3-.33		

SEARCH NOTES
(INCLUDING SEARCH STRATEGY)

EAST AIPB	Date	Exmr.
STN CAPLUS, MEDLINE, CANCERLIT, BIOSIS	4/7/01	JS
SMD		
reference		
target		
single molecule		
genomic equivalent		
molecular beacon		
loop hairpin		
stem		
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melting temperature		
updated	9/20/01	JS
updated	3/19/02	JS

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ISSUE SLIP STAPLE AREA (for additional cross references)

POSITION	INITIALS	ID NO.	DATE
FEE DETERMINATION	[illegible]	6550	7/20/2
O.I.P.E. CLASSIFIER	PH		9/22
FORMALITY REVIEW	AS	551	8-29-02
RESPONSE FORMALITY REVIEW	AS	573	03-22-01

INDEX OF CLAIMS

✓ ..... Rejected
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— (Through numeral)... Canceled
÷ ..... Restricted
N ..... Non-elected
I ..... Interference
A ..... Appeal
O ..... Objected

Claim Final	Claim Original	Date
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CONFIRMATION NO. 9893

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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

Verified and Acknowledged Examiner's Signature Initials

ADDRESS

22907

TITLE

Digital amplification

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BIBDATASHEET

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APPLICANTS

Bert Vogelstein, Baltimore, MD;

Kenneth W. Kinzler, BelAir, MD;

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

THIS APPLN CLAIMS BENEFIT OF 60/146,792 08/02/1999

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

IF REQUIRED, FOREIGN FILING LICENSE GRANTED ** SMALL ENTITY **
** 08/29/2000

		STATE OR	SHEETS	TOTAL	INDEPENDENT
Foreign Priority claimed	☐ yes ☐ no	COUNTRY	DRAWING	CLAIMS	CLAIMS
35 USC 119 (a-d) conditions met	☐ yes ☐ no ☐ Met after Allowance	MD	7	64	5
Verified and Acknowledged	Examiner's Signature Initials				

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TITLE
Digital amplification

FILING FEE	FEES: Authority has been given in Paper	☐ All Fees
RECEIVED	No. _____ to charge/credit DEPOSIT ACCOUNT	☐ 1.16 Fees (Filing)
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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.

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

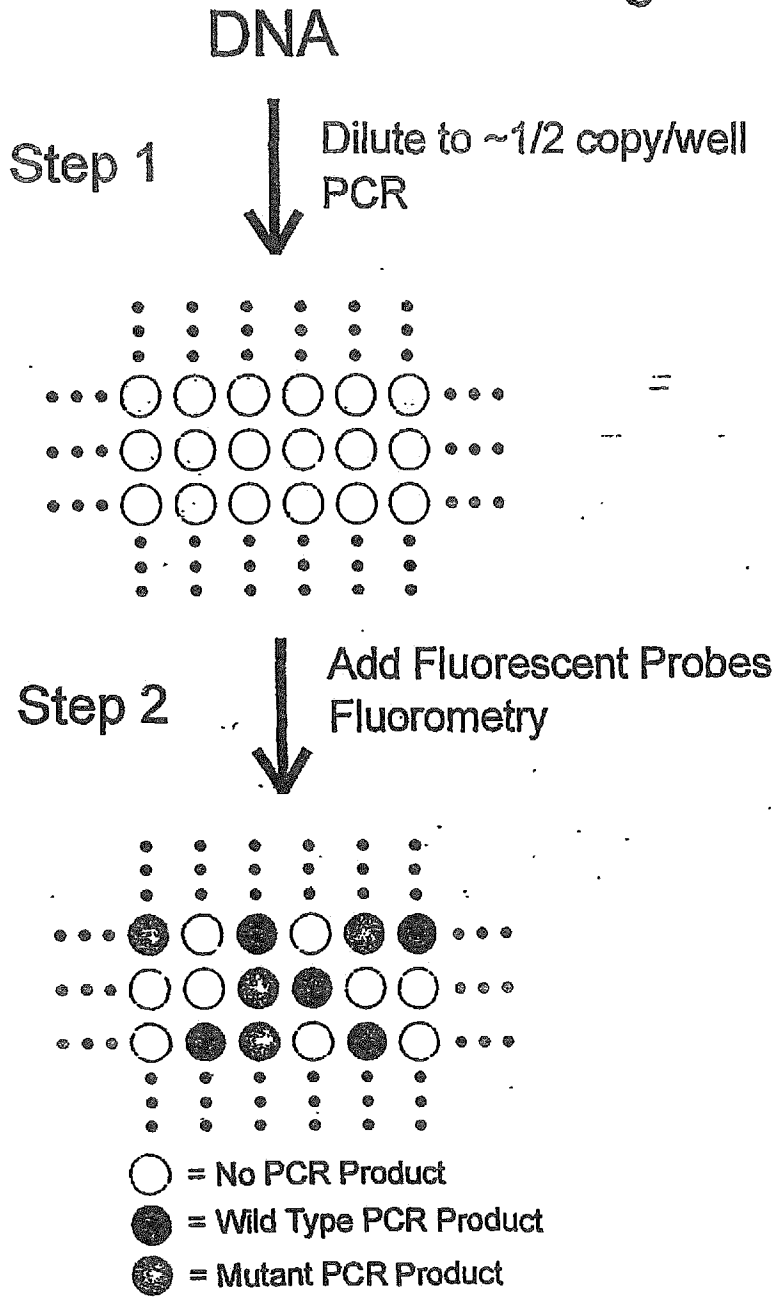

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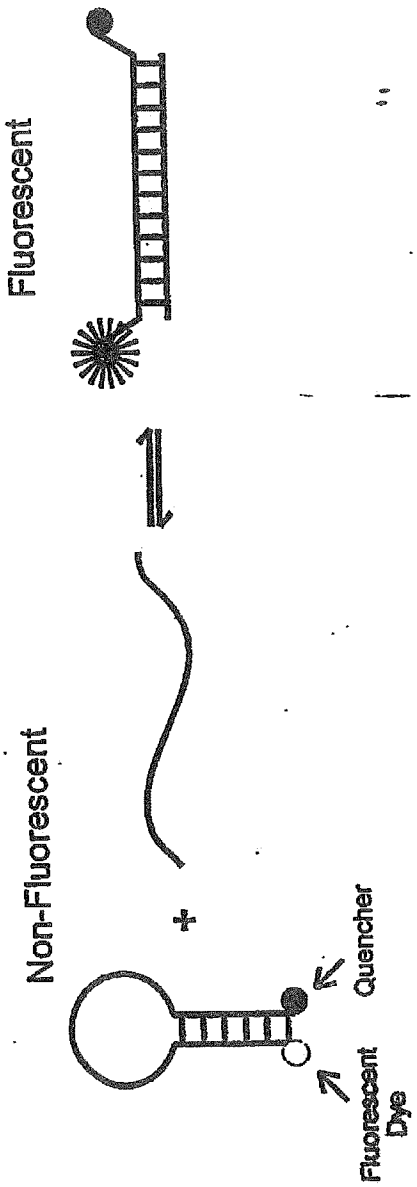
Fig. 1B



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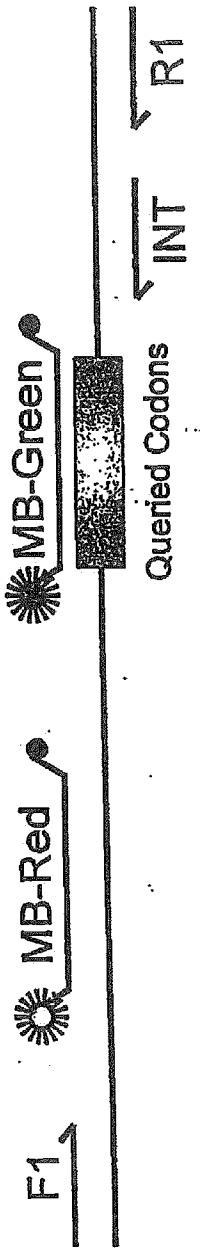
AS ORIGINALLY FILED

Fig. 1C



PRINT OF DRAWINGS
AS ORIGINALLY FILED

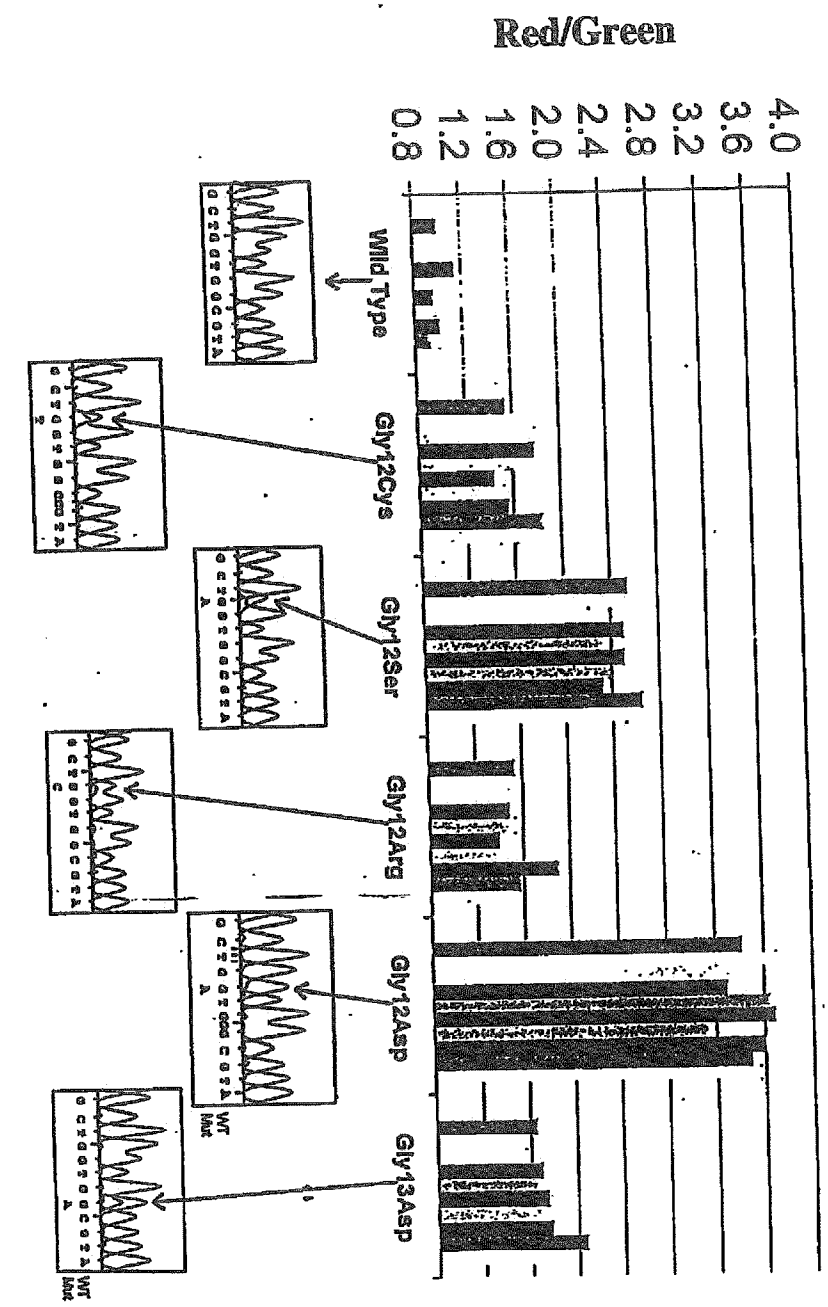
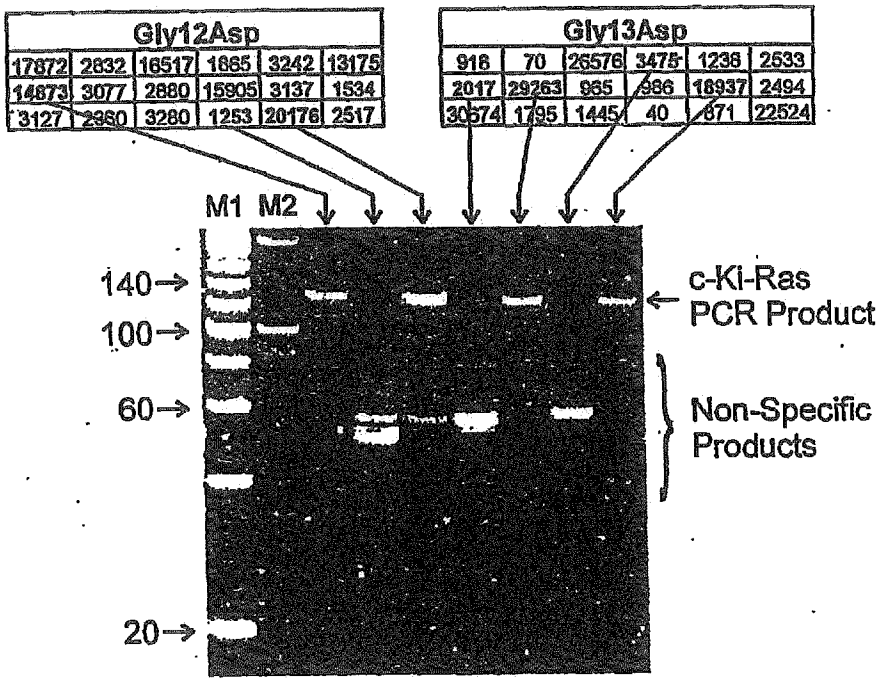


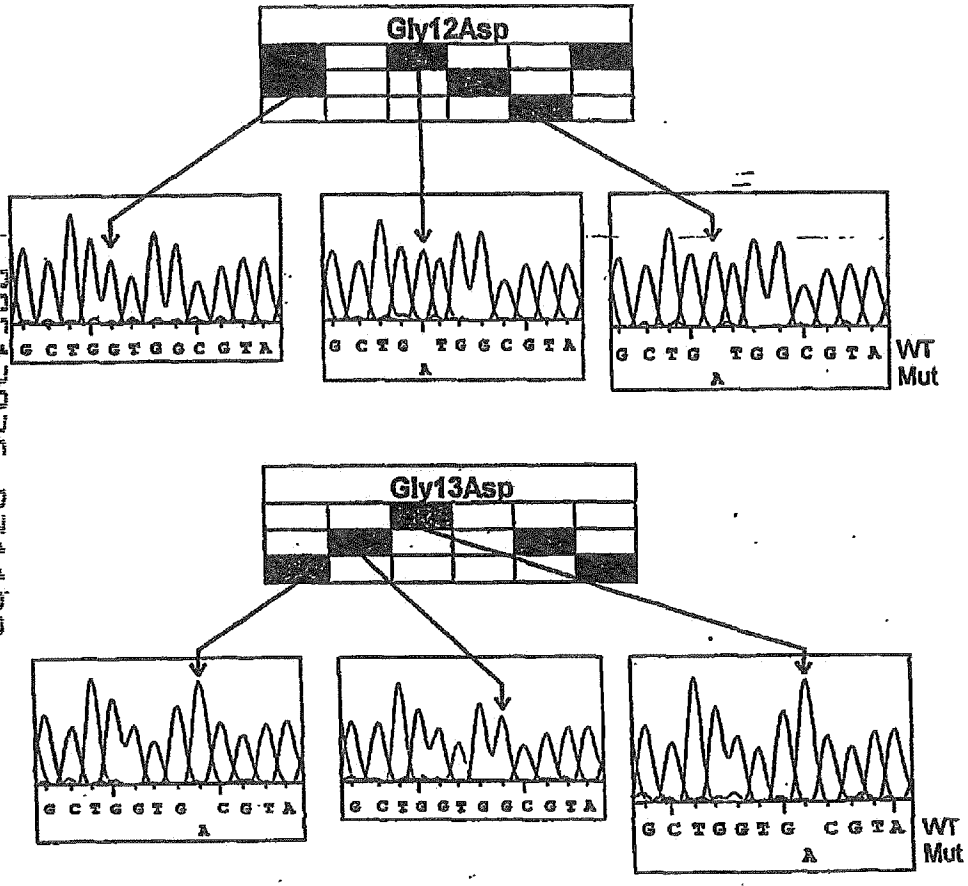
Fig. 2

Fig. 3



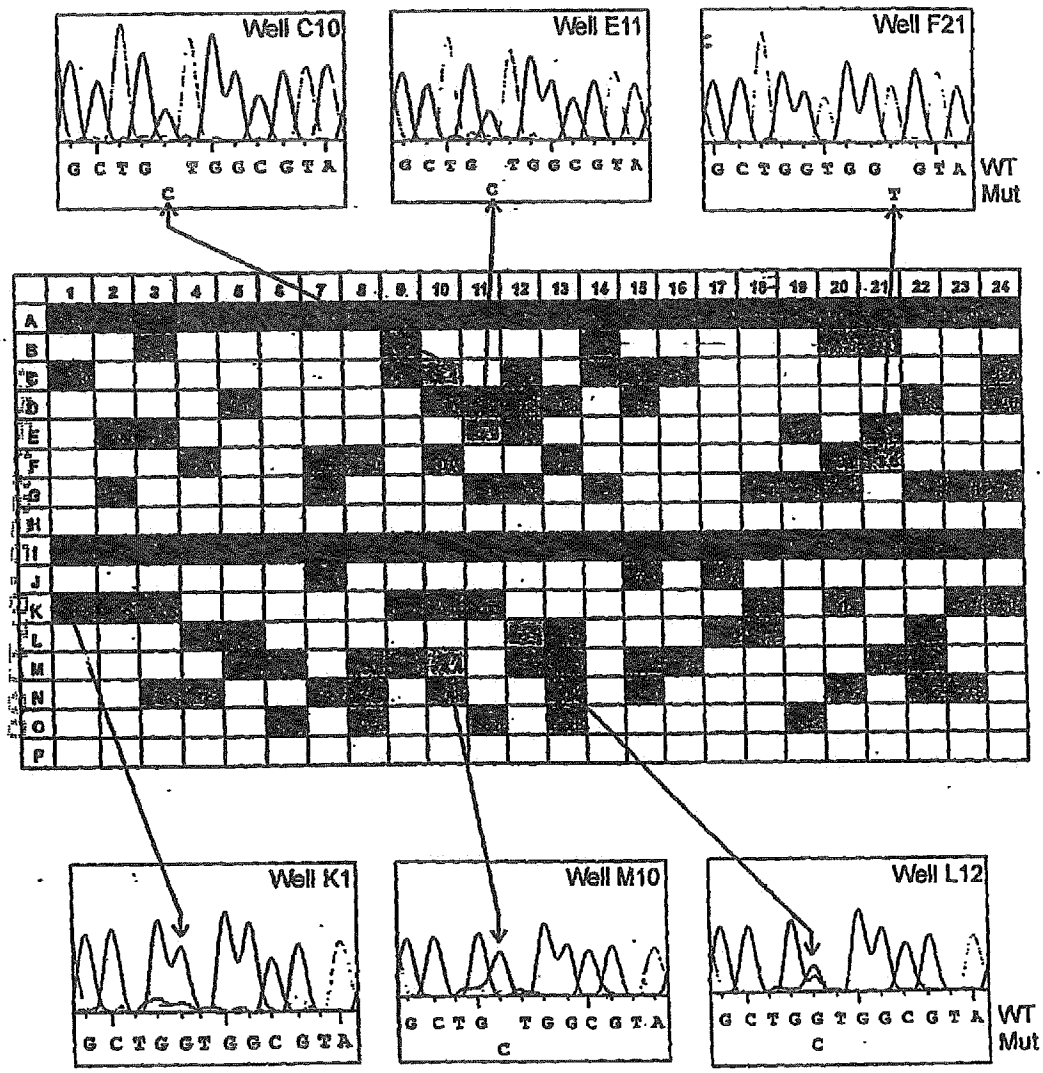
PRINT OF DRAWINGS
AS ORIGINALLY FILED

Fig. 4



PRINT OF DRAWINGS
AS ORIGINALLY FILED

Fig. 5



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

Atty. Docket No. 01107.00031

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

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

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

Title of Invention: DIGITAL AMPLIFICATION

1. ■ Specification 32 pages (including specification, claims, abstract) / 64 claims (5 independent)

2. ■ Declaration/Power of Attorney is:
   - ☐ attached in the regular manner.
   - ■ NOT included, but deferred under 37 C.F.R. § 1.53(f).

3. ■ 7 Distinct sheets of ■ Formal ☐ Informal Drawings

4. ☐ Preliminary Amendment.

5. ☐ Information Disclosure Statement
   - ☐ Form 1449
   - ☐ A copy of each cited prior art reference

6. ☐ Assignment with Cover Sheet.

7. ☐ Priority is hereby claimed under 35 U.S.C. § 119 based upon the following application(s):

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

8. ☐ Priority document(s).

9. ☐ Statement Claiming Small Entity Status.

10. ☐ Microfiche Computer Program (Appendix).

11. ☐ Nucleotide and/or Amino Acid Sequence Submission.
    - ☐ Computer Readable Copy.
    - ☐ Paper Copy (identical to computer copy).
    - ☐ Statement verifying identity of above copies.

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

12. Calculation of Fees:

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

13. PAYMENT is:

☐ included in the amount of the GRAND TOTAL by our enclosed check. A general authorization under 37 C.F.R. § 1.25(b), second sentence, is hereby given to credit or debit our Deposit Account No. 19-0733 for the instant filing and for any other fees during the pendency of this application under 37 C.F.R. §§ 1.16, 1.17 and 1.18.

■ not included, but deferred under 37 C.F.R. § 1.53(f).

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

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

15. Other: _____

Date: July 11, 2000

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

SAK/ama

#2

UNITED STATES PATENT AND TRADEMARK OFFICE

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

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

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

FORMALITIES LETTER

OC000000005521419

Date Mailed: 11/01/2000

NOTICE TO FILE MISSING PARTS OF NONPROVISIONAL APPLICATION

FILED UNDER 37 CFR 1.53(b)

Filing Date Granted

An application number and filing date have been accorded to this application. The item(s) indicated below, however, are missing. Applicant is given TWO MONTHS from the date of this Notice within which to file all required items and pay any fees required below to avoid abandonment. Extensions of time may be obtained by filing a petition accompanied by the extension fee under the provisions of 37 CFR 1.136(a).

- The statutory basic filing fee is missing.
  Applicant must submit $ 690 to complete the basic filing fee and/or file a small entity statement claiming such status (37 CFR 1.27).
- Total additional claim fee(s) for this application is $948.
  - $792 for 44 total claims over 20.
  - $156 for 2 independent claims over 3 .
- The oath or declaration is missing.
  A properly signed oath or declaration in compliance with 37 CFR 1.63, identifying the application by the above Application Number and Filing Date, is required.
- To avoid abandonment, a late filing fee or oath or declaration surcharge as set forth in 37 CFR 1.16(e) of $130 for a non-small entity, must be submitted with the missing items identified in this letter.

- **The balance due by applicant is $ 1768.**

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

Customer Service Center
Initial Patent Examination Division (703) 308-1202
PART 3 - OFFICE COPY

SECTOR

PATENT

#3

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

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

FOR: DIGITAL AMPLIFICATION

SUBMISSION OF EXECUTED DECLARATION
FOR PATENT APPLICATION AND FILING FEES

Assistant Commissioner for Patents
Washington, D.C. 20231

Sir:

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

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

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

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

Respectfully submitted,

Date: December 12, 2000

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

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

(202) 508-9100
SAK/ama

LARATION FOR PATENT APP. CATION

d inventor, we hereby declare that:

Our resid. 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.**

09613826
190733
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355.00 CH
90.00 CH
396.00 CH
52.00 CH
SMUMS
12/19/2000
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

#5

Sheet 1 of 1

PTO-1449 (Modified) U.S. DEPARTMENT OF COMMERCE PATENT AND TRADEMARK OFFICE INFORMATION DISCLOSURE STATEMENT BY APPLICANT	ATTY. DOCKET NO. 01107.00031	SERIAL NUMBER 09/613,926 ~~TBA~~
	APPLICANTS Bert Vogelstein, *et al.*	
	FILING DATE July 11, 2000	GROUP ART UNIT ~~TBA~~ 1637

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 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.

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 61382 ~~09/615,826~~
	APPLICANT Bert Vogelstein et al.	
	FILING DATE July 11, 2000	GROUP ART UNIT 1637

OIPE JUL 12 2001 PATENT & TRADEMARK OFFICE JC21

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 reproduction Vol. 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
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A

☒ PATENT ☐ DESIGN B&W Ref. 1107.00031 Date 5/7/01
☐ HAND CARRY Group/Section _____ Bldg _____ Rm _____
Serial/Patent No. 09/513,926 Atty/Sec SAK/sf
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# _____
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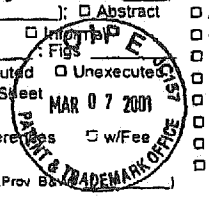

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PATENT APPLICATION

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re application of:	)	
	)	
Bert Vogelstein, *et al.*	)	Group Art No. 1632
	)	
Serial No.: 09/613,826	)	Examiner: TBA
	)	
Filed: July 11, 2000	)	Docket No. 01107.00031

For: DIGITAL AMPLIFICATION

INFORMATION DISCLOSURE STATEMENT

Assistant Commissioner for Patents
Washington, D.C. 20231

Sir:

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

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

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

Respectfully submitted,

BANNER & WITCOFF, LTD.

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

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

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
	Honghua LI, *et al.*, "Amplification and Analysis of DNA Sequences in Single Human Sperm and Diploid Cells", Nature, Vol. 335, September 29, 1988
	Ramon PARSONS, *et al.*, "Mismatch Repair Deficiency in Phenotypically Normal Human Cells", Science, Vol. 268, May 5, 1995
	Lin ZHANG, *et al.*, "Whole Genome Amplification from a Single Cell: Implications for Genetic Analysis", Proc. National Science USA, Vol. 89, pp. 5847-5851, July 1992
	David SIDRANSKY, *et al.*, "Clonal Expansion of p53 Mutant Cells is Associated with Brain Tumour Progression", Nature, February 27, 1992
	Alec J. Jeffreys, *et al.*, "Mutation Processes at Human Minisatellites", Electophoresis, pp. 1577-1585, 1995
	C. SCHMITT, *et al.*, "High Sensitive DNA Typing Approaches for the Analysis of Forensic Evidence: Comparison of Nested Variable Number of Tandem Repeats (VNTR) Amplification and Short Tandem Repeats (STR) Polymorphism", Forensic Science International, Vol. 66, pp. 129-141, 1994
	Paul M. LIZARDI, *et al.*, "Mutation Detection and Single-Molecule Counting Using Isothermal Rolling-Circle Amplification", Nature Genetics, Vol. 19, July 1998
	W. NAVIDI, *et al.*, "Using PCR in Preimplantation Genetic Disease Diagnosis", Human Reproduction, Vol. 6, 1991
JS	Honghua LI, *et al.*, "Amplification and Analysis of DNA Sequences in Single Human Sperm and Diploid Cells" Nature, Vol. 335, September 29, 1988

EXAMINER	DATE CONSIDERED
Jeffrey Siew	3/23/02

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

B

Patent/Design ☐ PATENT ☐ DESIGN

☐ HAND CARRY Group/Section _____ Bldg _____ Rm ____

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

Inventor [illegible] Client JHU

Title Digital Manipulation

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

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☐ Amendment ☐ Response : OA dtd _____

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☐ Brief : ☐ Appeal & Fee ☐ Reply

Patent/Design ☐ PATENT ☐ DESIGN

☐ HAND CARRY Group/Section _____ Bldg _____ Rm ____

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

Inventor [illegible] Client JHU

Title Digital Manipulation

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# _____

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☐ Claim for Priority w/Priority Doc _____ Country, Appl # and Date

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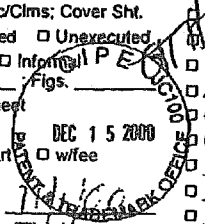

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TRANSMITTAL FORM

(to be used for all correspondence after initial filing)

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

Total Number of Pages in This Submission: 27

ENCLOSURES *(check all that apply)*

- ☐ Fee Transmittal Form
  - ☐ Fee Attached
- ☒ Amendment / Response
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  - ☐ 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
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- ☐ 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: ____

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SEQUENCE LISTING

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Kinzler, Kenneth W.

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PATENT

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re Application of

Bert Vogelstein, et. al.

Serial No. 09/613,826

Filing Date: July 11, 2000

) Group Art Unit: 1656

) Examiner: I Siew

) Docket No. 01107.00031

For: DIGITAL AMPLIFICATION

SUPPLEMENTAL SUBMISSION

Assistant Commissioner for Patents
Washington, D.C. 20231

Sir:

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

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

Respectfully submitted,

Date: July 17, 2001

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

BANNER & WITCOFF, LTD.
1001 G STREET, NW
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TRANSMITTAL FORM

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Application Number	09/613,826
Filing Date	July 11, 2000
First Named Inventor	VOGELSTEIN et al.
Group Art Unit	1656
Examiner Name	J. SIEW
Total Number of Pages in This Submission: 6	Attorney Docket Number: 01107.00031

ENCLOSURES *(check all that apply)*

- ☐ Fee Transmittal Form
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- ☐ Amendment / Response
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- ☐ Response to Missing Parts/ Incomplete Application
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- ☐ Drawing(s)
- ☐ Licensing-related Papers
- ☐ Petition Routing Slip (PTO/SB/69) and Accompanying Petition
- ☐ Petition to Convert to a Provisional Application
- ☐ Power of Attorney, Revocation Change of Correspondence Address
- ☐ Terminal Disclaimer
- ☐ Request for Refund
- ☐ CD, Number of CD(s) ____
- ☐ After Allowance Communication to Group
- ☐ Appeal Communication to Board of Appeals and Interferences
- ☐ Appeal Communication to Group *(Appeal Notice, Brief, Reply Brief)*
- ☐ Proprietary Information
- ☐ Status Letter
- ☒ Other Enclosure(s) *(please identify below)*:

  Supplemental Submission

  Copy of Reference

Remarks

SIGNATURE OF APPLICANT, ATTORNEY, OR AGENT

Firm or Individual name	Michelle Holmes-Son Reg. No. 47,680
Signature	Michelle Holmes-Son
Date	July 17, 2001

CERTIFICATE OF MAILING

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

Typed or printed name			
Signature		Date	

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 send 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.

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/.00001

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

BM12/0920

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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Kinzler, Kenneth W.
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<141> CURRENT FILING DATE: 2000-07-11
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<151> PRIOR FILING DATE: 1999-08-02
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PATENT APPLICATION: US/09/613,826 TIME: 11:12:17

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File History Report

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☒ The following page(s) 3 of 3 of paper number 11 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:_____

S. Siew — Re-run

#12 DHW 10-3-01

1656

OIPE

RAW SEQUENCE LISTING DATE: 09/20/2001
PATENT APPLICATION: US/09/613,826 TIME: 17:06:04

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

MESSAGE SUMMARY

#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 JS

IN THE CLAIMS

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

33. (Amended) A molecular beacon probe comprising:

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

36. (Amended) A molecular beacon probe comprising:

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

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

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

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

wherein the first and the second photoluminescent dyes are distinct.

65. (Amended) A molecular beacon probe comprising:

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

68. (Amended) A molecular beacon probe comprising:

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

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

a first oligonucleotide comprising a first stem and a first loop structure and having a photoluminescent dye at one of the 5' or 3' ends and a quenching agent at the opposite 5' or 3' end, wherein the first loop consists of 14-26 bases and has a Tm of 50-51°C, and wherein the first stem consists of 4 base pairs having a sequence 5'-CACG-3'; and

a second oligonucleotide comprising a second stem and a second loop structure and having a photoluminescent dye at one of the 5' or 3' ends and a quenching agent at the opposite 5' or 3' end, wherein the second loop consists of 14-26 bases and has a Tm

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

REMARKS

The Invention

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

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

The Amendments

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

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

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

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

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

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

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

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

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

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

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

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

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

Claims 65-69 have been rejected under 35 U.S.C. § 112, second paragraph, as being indefinite for failing to particularly point out and distinctly claim the subject matter which applicant regards as the invention. Specifically, the Office Action asserts that the phrase "comprising 19-20" renders claim 68 unclear. Claim 68 has been amended to

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

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

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

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

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

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

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

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

Respectfully submitted,

Date: December 6, 2001

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

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

MARKED UP VERSION OF THE CLAIMS TO SHOW CHANGES MADE

33. (Amended) A molecular beacon probe comprising:

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

36. (Amended) A molecular beacon probe comprising:

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

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

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

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

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

wherein the first and the second photoluminescent dyes are distinct.

65. (Amended) A molecular beacon probe comprising:

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

68. (Amended) A molecular beacon probe comprising:

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

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

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

a Tm of 50-51°C, and wherein the first stem [comprises] consists of 4 base pairs having a sequence 5'-CACG-3'; and

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

AF/1656

PTO/SB/21 (08-00)

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TRANSMITTAL FORM

(to be used for all correspondence after initial filing)

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

Total Number of Pages in This Submission: 13

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ENCLOSURES *(check all that apply)*

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

Remarks

SIGNATURE OF APPLICANT, ATTORNEY, OR AGENT

Firm or Individual name	Michelle Holmes-Son, Registration No. 47,660
Signature	Michelle Holmes-Son
Date	December 6, 2001

CERTIFICATE OF MAILING

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

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Signature		Date	

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

22907 7590 12/12/2001

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WASHINGTON, DC 20001

EXAMINER	
SIEW, JEFFREY	

ART UNIT	PAPER NUMBER
1656	14

DATE MAILED: 12/12/2001

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

PTO-90C (Rev. 07-01)

copy

Advisory Action	Application No.	Applicant(s)	
	09/613,826	VOGELSTEIN ET AL.	
	Examiner	Art Unit	
	Jeffrey Siew	1656	

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

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

PERIOD FOR REPLY [check either a) or b)]

a) ☒ The period for reply expires 3 months from the mailing date of the final rejection.

b) ☐ The period for reply expires on: (1) the mailing date of this Advisory Action, or (2) the date set forth in the final rejection, whichever is later. In no event, however, will the statutory period for reply expire later than SIX MONTHS from the mailing date of the final rejection. ONLY CHECK THIS BOX WHEN THE FIRST REPLY WAS FILED WITHIN TWO MONTHS OF THE FINAL REJECTION. See MPEP 706.07(f).

Extensions of time may be obtained under 37 CFR 1.136(a). The date on which the petition under 37 CFR 1.136(a) and the appropriate extension fee have been filed is the date for purposes of determining the period of extension and the corresponding amount of the fee. The appropriate extension fee under 37 CFR 1.17(a) is calculated from: (1) the expiration date of the shortened statutory period for reply originally set in the final Office action; or (2) as set forth in (b) above, if checked. Any reply received by the Office later than three months after the mailing date of the final rejection, even if timely filed, may reduce any earned patent term adjustment. See 37 CFR 1.704(b).

1. ☐ A Notice of Appeal was filed on _____. Appellant's Brief must be filed within the period set forth in 37 CFR 1.192(a), or any extension thereof (37 CFR 1.191(d)), to avoid dismissal of the appeal.

2. ☒ The proposed amendment(s) will not be entered because:

   (a) ☒ they raise new issues that would require further consideration and/or search (see NOTE below);

   (b) ☐ they raise the issue of new matter (see Note below);

   (c) ☐ they are not deemed to place the application in better form for appeal by materially reducing or simplifying the issues for appeal; and/or

   (d) ☐ they present additional claims without canceling a corresponding number of finally rejected claims.

   NOTE: *the limitation to consisting of 14-26 would require new search and consideration*.

3. ☒ Applicant's reply has overcome the following rejection(s): *See Continuation Sheet*.

4. ☐ Newly proposed or amended claim(s) _____ would be allowable if submitted in a separate, timely filed amendment canceling the non-allowable claim(s).

5. ☐ The a)☐ affidavit, b)☐ exhibit, or c)☐ request for reconsideration has been considered but does NOT place the application in condition for allowance because: _____.

6. ☐ The affidavit or exhibit will NOT be considered because it is not directed SOLELY to issues which were newly raised by the Examiner in the final rejection.

7. ☐ For purposes of Appeal, the proposed amendment(s) a)☐ will not be entered or b)☐ will be entered and an explanation of how the new or amended claims would be rejected is provided below or appended.

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

   Claim(s) allowed: *1-64*.

   Claim(s) objected to: _____.

   Claim(s) rejected: *65-69*.

   Claim(s) withdrawn from consideration: _____.

8. ☐ The proposed drawing correction filed on _____ is a)☐ approved or b)☐ disapproved by the Examiner.

9. ☐ Note the attached Information Disclosure Statement(s)(PTO-1449) Paper No(s). _____.

10. ☐ Other: _____

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

Jeffrey Lew
3/23/02
copy filed 12/13/01

PATENT APPLICATION

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

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

INFORMATION DISCLOSURE STATEMENT

Assistant Commissioner for Patents
Washington, D.C. 20231

Sir:

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

Each item of information contained in the information disclosure statement was first cited in any communication from a foreign patent office in a counterpart foreign application not more than three months prior to the filing of the information disclosure statement.

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

Respectfully submitted,

Date: January 23, 2002

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

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

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INFORMATION DISCLOSURE STATEMENT BY APPLICANT

(use as many sheets as necessary)

Sheet 1 of

JAN 23 2002 PATENT & TRADEMARK OFFICE

Complete If Known	
Application Number	09/613,826
Filing Date	July 11, 2000
First Named Inventor	Bert Vogelstein et al.
Group Art Unit	1656 1637
Examiner Name	J. Slew
Attorney Docket Number	01107.00031

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U.S. PATENT DOCUMENTS

Examiner Initials*	Cite No.[1]	Document Number Number - Kind Code[2] (if known)	Publication Date MM-DD-YYYY	Name of Patentee or Applicant of Cited Document	Pages, Columns, Lines, Where Relevant Passages or Relevant Figures Appear
JS		US-5,804,383	09-08-1998	Gruenert et al.	
JS		US- 5,658,663	01-12-1999	Nisson et al.	
		US-			
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FOREIGN PATENT DOCUMENTS

Examiner Initials*	Cite No.[1]	Foreign Patent Document Country Code[3] - Number[4] - Kind Code[5] (if known)	Publication Date MM-DD-YYYY	Name of Patentee or Applicant of Cited Document	Pages, Columns, Lines, Where Relevant Passages or Relevant Figures Appear	T[6]
JS		WO 95/13399	05-18-1995			
		EP 0643140 A	03-15-1995			
JS		WO 99/13113	03-18-1999			

Examiner Signature	Jeffrey Siew	Date Considered	3/23/02

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

[1] Applicant's unique citation designation number (optional). [2] See Kinds Codes of USPTO Patent Documents at www.uspto.gov or MPEP 901.04. [3] Enter Office that issued the document, by the two-letter code (WIPO Standard ST.3). [4] For Japanese patent documents, the indication of the year of the reign of the Emperor must precede the serial number of the patent document. [5] Kind of document by the appropriate symbols as indicated on the document under WIPO Standard ST. 16 if possible. [6] Applicant is to place a check mark here if English language Translation is attached.

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INFORMATION DISCLOSURE STATEMENT BY APPLICANT

(use as many sheets as necessary)

Sheet | of | 2

Complete if Known	
Application Number	09/613,826
Filing Date	July 11, 2000
First Named Inventor	Bert Vogelstein et al.
Group Art Unit	~~1635~~ 1637
Examiner Name	J. Siew
Attorney Docket Number	01107.00031

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OTHER PRIOR ART -- NON PATENT LITERATURE DOCUMENTS

Examiner Initials *	Cite No.[1]	Include name of the author (in CAPITAL LETTERS), title of the article (when appropriate), title of the item (book, magazine, journal, serial, symposium, catalog, etc.), date, page(s), volume-issue number(s), publisher, city and/or country where published.	T[2]
		A. PIATEK et al., "Molecular Beacon Sequence Analysis for Detecting Drug Resistance in Mycobacterium Tuberculosis", Nature Biotechnology, April 1998, pp. 359-363, Vol. 16, No. 4	
		S. TYAGI et al., "Multicolor Molecular Beacons for allele discrimination", Nature Biotechnology, pp. 303-308, January 1998, Vol. 16, No. 1	
		J. A.M. VET et al., "Multiplex Detection of Four Pathogenic Retroviruses Using Molecular Beacons", Proceedings of the National Academy of Sciences of the United States", May 25, 1999, pp. 6394-6399, Vol. 96, No. 11	
		S. TYAGI et al., "Molecular Beacons: probes that Fluoresce Upon Hybridization", Nature Biotechnology, 1996, pp. 303-308, Vol. 14, No. 3	
		W. P. HALFORD et al., "The Inherent Quantitative Capacity of the Reverse Transcription-Polymerase Chain Reaction", Analytical Biochemistry, January 15, 1999, pp. 181-191, Vol. 266, No. 2	
		B. VOGELSTEIN et al., "Digital PCR", Proceedings of the National Academy of Sciences of the United States, August 3, 1999, pp. 9236-9241, Vol. 96, No. 16	
		K. D.E. EVERETT et al, "Identification of nine species of the Chlamydiaceae Using PCR-RFLP", April 1999, pp. 803-813, Vol. 49, No. 2	

Examiner Signature	Jeffrey Siew	Date Considered	3/18/02

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

[1] Unique citation designation number (optional). [2] Applicant is to place a check mark here if English language Translation is attached.

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FEE TRANSMITTAL for FY 2002

Patent fees are subject to annual revision.

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TOTAL AMOUNT OF PAYMENT ($) 180

Complete If Known	
Application Number	09/613,826
Filing Date	July 11, 2000
First Named Inventor	Bert Vogelstein et al.
Examiner Name	J. Siew
Group / Art Unit	1656
Attorney Docket No.	01107.00031

METHOD OF PAYMENT (check all that apply)

☐ Check ☐ Credit card ☐ Money Order ☐ Other ☐ None

☒ Deposit Account:

Deposit Account Number: Banner & Witcoff, Ltd.

Deposit Account Name: 19-0733

The Commissioner is authorized to: *(check all that apply)*
☒ Charge fee(s) indicated below ☒ Credit any overpayments
☒ Charge any additional fee(s) during the pendency of this application
☐ Charge fee(s) indicated below, except for the filing fee to the above-identified deposit account.

FEE CALCULATION

1. BASIC FILING FEE

Large Entity Fee Code	Large Entity Fee ($)	Small Entity Fee Code	Small Entity Fee ($)	Fee Description	Fee Paid
101	740	201	370	Utility filing fee	
106	330	206	165	Design filing fee	
107	510	207	255	Plant filing fee	
108	740	208	370	Reissue filing fee	
114	160	214	80	Provisional filing fee	

SUBTOTAL (1) ($) 0

2. EXTRA CLAIM FEES

			Extra Claims		Fee from below	=	Fee Paid
Total Claims		-20 **	= 0	X		=	0
Independent Claims		-3 **	= 0	X		=	0
Multiple Dependent				X		=	0

Large Entity Fee Code	Large Entity Fee ($)	Small Entity Fee Code	Small Entity Fee ($)	Fee Description
103	18	203	9	Claims in excess of 20
102	84	202	42	Independent claims in excess of 3
104	280	204	140	Multiple dependent claim, if not paid
109	84	209	42	** Reissue independent claims over original patent
110	18	210	9	** Reissue claims in excess of 20 and over original patent

SUBTOTAL (2) ($) 0

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

FEE CALCULATION (continued)

3. ADDITIONAL FEES

Large Entity Fee Code	Large Entity Fee ($)	Small Entity Fee Code	Small Entity Fee ($)	Fee Description	Fee Paid
105	130	205	65	Surcharge - late filing fee or oath	
127	50	227	25	Surcharge - late provisional filing fee or cover sheet.	
139	130	139	130	Non-English specification	
147	2,520	147	2,520	For filing a request for reexamination	
112	920*	112	920*	Requesting publication of SIR prior to Examiner action	
113	1,840*	113	1,840*	Requesting publication of SIR after Examiner action	
115	110	215	55	Extension for reply within first month	
116	400	216	200	Extension for reply within second month	
117	920	217	460	Extension for reply within third month	
118	1,440	218	720	Extension for reply within fourth month	
128	1,960	228	980	Extension for reply within fifth month	
119	320	219	160	Notice of Appeal	
120	320	220	160	Filing a brief in support of an appeal	
121	280	221	140	Request for oral hearing	
138	1,510	138	1,510	Petition to institute a public use proceeding	
140	110	240	55	Petition to revive – unavoidable	
141	1,280	241	640	Petition to revive – unintentional	
142	1,280	242	640	Utility issue fee (or reissue)	
143	460	243	230	Design issue fee	
144	620	244	310	Plant issue fee	
122	130	122	130	Petitions to the Commissioner	
123	50	123	50	Processing fee under 37 CFR 1.17 (q)	
126	180	126	180	Submission of Information Disclosure Stmt	180
581	40	581	40	Recording each patent assignment per property (times number of properties)	
148	740	248	370	Filing a submission after final rejection (37 CFR § 1.129(a))	
149	740	249	370	For each additional invention to be examined (37 CFR § 1.129(b))	
179	740	279	370	Request for Continued Examination (RCE)	
169	900	169	900	Request for expedited examination of a design application	
				Other fee (specify)	

*Reduced by Basic Filing Fee Paid SUBTOTAL (3) ($) 180

SUBMITTED BY				Complete (if applicable)	
Name (Print/Type)	Michelle L. Holmes-Son	Registration No. Attorney/Agent	47,660	Telephone	202-508-9100
Signature	Michelle L. Holmes-Son			Date	January 23, 2002

WARNING: Information on this form may become public. Credit card information should not be included on this form. Provide credit card information and authorization on PTO-2038.

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

1656

PTO/SB/21 (08-00)

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

Approved for use through 10/31/2002. OMB 0651-0031
U.S. Patent and Trademark Office: U.S. DEPARTMENT OF COMMERCE
Under the Paperwork Reduction Act of 1995, no persons are required to respond to a collection of information unless it displays a valid OMB control number.

TRANSMITTAL FORM

(to be used for all correspondence after initial filing)

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

Total Number of Pages in This Submission

RECEIVED
JAN 28 2002
TECH CENTER 1600/2900

ENCLOSURES *(check all that apply)*

- [x] Fee Transmittal Form
- [ ] Fee Attached
- [ ] Amendment / Response
- [ ] After Final
- [ ] Affidavits/declaration(s)
- [ ] Extension of Time Request
- [ ] Express Abandonment Request
- [x] Information Disclosure Statement
- [ ] Certified Copy of Priority Document(s)
- [ ] Response to Missing Parts/ Incomplete Application
- [ ] Response to Missing Parts under 37 CFR 1.52 or 1.53
- [ ] Assignment Papers *(for an Application)*
- [ ] Drawing(s)
- [ ] Licensing-related Papers
- [ ] Petition
- [ ] Petition to Convert to a Provisional Application
- [ ] Power of Attorney, Revocation Change of Correspondence Address
- [ ] Terminal Disclaimer
- [ ] Request for Refund
- [ ] CD, Number of CD(s) ____
- [ ] After Allowance Communication to Group
- [ ] Appeal Communication to Board of Appeals and Interferences
- [ ] Appeal Communication to Group *(Appeal Notice, Brief, Reply Brief)*
- [ ] Proprietary Information
- [ ] Status Letter
- [x] Other Enclosure(s) *(please identify below)*: PTO-1449 (w/references)

Remarks

SIGNATURE OF APPLICANT, ATTORNEY, OR AGENT

Firm or Individual name	Michelle L. Holmes-Son, Registration No. 47,660
Signature	Michelle L Holmes-Son
Date	January 23, 2002

CERTIFICATE OF MAILING

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

Typed or printed name			
Signature		Date	

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

PTO/SB/22 (10-00)
Approved for use through 10/31/2002. OMB 0651-0031
U.S. Patent and Trademark Office: U.S. DEPARTMENT OF COMMERCE
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.

PETITION FOR EXTENSION OF TIME UNDER 37 CFR 1.136(a)

Docket Number (Optional): 001107.00031

In re Application of Bert VOGELSTEIN, et a.

Application Number 09/613,826 | Filed July 11, 2000

For Digital Amplification

Group Art Unit 1656 | Examiner Jeffrey Siew

OIPE JC16 FEB 20 2002 PATENT & TRADEMARK OFFICE

TECH CENTER 1600/2900 FEB 25 2002 RECEIVED

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

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

- ☐ One month (37 CFR 1.17(a)(1)) $____
- ☒ Two months (37 CFR 1.17(a)(2)) $400.00
- ☐ Three months (37 CFR 1.17(a)(3)) $____
- ☐ Four months (37 CFR 1.17(a)(4)) $____
- ☐ Five months (37 CFR 1.17(a)(5)) $____

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

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

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

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

☒ The Commissioner is hereby authorized to charge any fees which may be required, or credit any overpayment, to Deposit Account Number 19-0733 .
I have enclosed a duplicate copy of this sheet.

I am the ☐ applicant/inventor.

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

☒ attorney or agent of record.

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

WARNING: Information on this form may become public. Credit card information should not be included on this form. Provide credit card information and authorization on PTO-2038.

February 20, 2002
Date

Michelle L. Holmes-Son
Signature

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

02/26/2002 AWONDI 00000064 190733 09613826
01:21:16 200.00 CH

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

☐ *Total of ____ forms are submitted.

Burden Hour Statement: This form is estimated to take 0.1 hours to complete. Time will vary depending upon the needs of the individual case. Any comments on the amount of time you are required to complete this form should be sent to the Chief Information Officer, U.S. Patent and Trademark Office, Washington, DC 20231. DO NOT SEND FEES OR COMPLETED FORMS TO THIS ADDRESS. SEND TO: Assistant Commissioner for Patents, Washington, DC 20231.

Approved for use through 10/31/2002. OMB 0651-0032
U.S. Patent and Trademark Office: U.S. DEPARTMENT OF COMMERCE
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FEE TRANSMITTAL for FY 2002

OIPE FEB 20 2002 PATENT & TRADEMARK OFFICE

Patent fees are subject to annual revision.

RECEIVED FEB 25 2002 TECH CENTER 1600/2900

Complete If Known	
Application Number	09/813,926
Filing Date	July 11, 2000
First Named Inventor	Bert VOGELSTEIN, et al.
Examiner Name	Jeffrey Siew
Group / Art Unit	1656
Attorney Docket No.	001107 00031

TOTAL AMOUNT OF PAYMENT ($) 200

METHOD OF PAYMENT (check all that apply)

☐ Check ☐ Credit card ☐ Money Order ☐ Other ☐ None

☐ Deposit Account:

Deposit Account Number: 19-0733

Deposit Account Name: Banner & Witcoff, Ltd

The Commissioner is authorized to: *(check all that apply)*
☐ Charge fee(s) indicated below ☐ Credit any overpayments
☒ Charge any additional fee(s) during the pendency of this application
☐ Charge fee(s) indicated below, except for the filing fee
to the above-identified deposit account.

FEE CALCULATION

1. BASIC FILING FEE

Large Entity Fee Code	Large Entity Fee ($)	Small Entity Fee Code	Small Entity Fee ($)	Fee Description	Fee Paid
101	740	201	370	Utility filing fee	
106	330	206	165	Design filing fee	
107	510	207	255	Plant filing fee	
108	740	208	370	Reissue filing fee	
114	160	214	80	Provisional filing fee	

SUBTOTAL (1) ($) 0

2. EXTRA CLAIM FEES

			Extra Claims		Fee from below	=	Fee Paid
Total Claims		-20 **	0	X		=	0
Independent Claims		-3 **	0	X		=	0
Multiple Dependent				X		=	0

Large Entity Fee Code	Large Entity Fee ($)	Small Entity Fee Code	Small Entity Fee ($)	Fee Description
103	18	203	9	Claims in excess of 20
102	84	202	42	Independent claims in excess of 3
104	280	204	140	Multiple dependent claim, if not paid
109	84	209	42	** Reissue independent claims over original patent
110	18	210	9	** Reissue claims in excess of 20 and over original patent

SUBTOTAL (2) ($) 0

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

FEE CALCULATION (continued)

3. ADDITIONAL FEES

Large Entity Fee Code	Large Entity Fee ($)	Small Entity Fee Code	Small Entity Fee ($)	Fee Description	Fee Paid
105	130	205	65	Surcharge - late filing fee or oath	
127	50	227	25	Surcharge - late provisional filing fee or cover sheet.	
139	130	139	130	Non-English specification	
147	2,520	147	2,520	For filing a request for reexamination	
112	920*	112	920*	Requesting publication of SIR prior to Examiner action	
113	1,840*	113	1,840*	Requesting publication of SIR after Examiner action	
115	110	215	55	Extension for reply within first month	
116	400	216	200	Extension for reply within second month	200.00
117	920	217	460	Extension for reply within third month	
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128	1,960	228	980	Extension for reply within fifth month	
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120	320	220	160	Filing a brief in support of an appeal	
121	280	221	140	Request for oral hearing	
138	1,510	138	1,510	Petition to institute a public use proceeding	
140	110	240	55	Petition to revive – unavoidable	
141	1,280	241	640	Petition to revive – unintentional	
142	1,280	242	640	Utility issue fee (or reissue)	
143	460	243	230	Design issue fee	
144	620	244	310	Plant issue fee	
122	130	122	130	Petitions to the Commissioner	
123	50	123	50	Processing fee under 37 CFR 1.17 (q)	
126	180	126	180	Submission of Information Disclosure Stmt	
581	40	581	40	Recording each patent assignment per property (times number of properties)	
146	740	246	370	Filing a submission after final rejection (37 CFR § 1.129(a))	
149	740	249	370	For each additional invention to be examined (37 CFR § 1.129(b))	
179	740	279	370	Request for Continued Examination (RCE)	
169	900	169	900	Request for expedited examination of a design application	
Other fee (specify) ____					

*Reduced by Basic Filing Fee Paid SUBTOTAL (3) ($) 200

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

WARNING: Information on this form may become public. Credit card information should not be included on this form. Provide credit card information and authorization on PTO-2038.

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

AF/1656 8x

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	Bert VOGELSTEIN, et al.
Group Art Unit	1656
Examiner Name	Jeffrey Siew
Attorney Docket Number	001107.00031

Total Number of Pages in This Submission

ENCLOSURES *(check all that apply)*

- ☒ Fee Transmittal Form
  - ☐ Fee Attached
- ☒ Amendment / Response
  - ☒ After Final
  - ☐ Affidavits/declaration(s)
- ☒ Extension of Time Request
- ☐ Express Abandonment Request
- ☐ Information Disclosure Statement
- ☐ Certified Copy of Priority Document(s)
- ☐ Response to Missing Parts/ Incomplete Application
  - ☐ Response to Missing Parts under 37 CFR 1.52 or 1.53
- ☐ Assignment Papers *(for an Application)*
- ☐ Drawing(s)
- ☐ Licensing-related Papers
- ☐ Petition
- ☐ Petition to Convert to a Provisional Application
- ☐ Power of Attorney, Revocation Change of Correspondence Address
- ☐ Terminal Disclaimer
- ☐ Request for Refund
- ☐ CD, Number of CD(s) ____
- ☐ After Allowance Communication to Group
- ☐ Appeal Communication to Board of Appeals and Interferences
- ☐ Appeal Communication to Group *(Appeal Notice, Brief, Reply Brief)*
- ☐ Proprietary Information
- ☐ Status Letter
- ☒ Other Enclosure(s) *(please identify below)*:
  - Sequence Listing
  - Computer Readable Disk

Remarks

SIGNATURE OF APPLICANT, ATTORNEY, OR AGENT

Firm or Individual name	Michelle L. Holmes-Son, Reg. No. 47,860
Signature	Michelle L. Holmes-Son
Date	February 20, 2002

CERTIFICATE OF MAILING

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

Typed or printed name			
Signature		Date	

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

PATENT

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

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

For: **DIGITAL AMPLIFICATION**

AMENDMENT AFTER FINAL REJECTION

Assistant Commissioner for Patents
Washington, D.C. 20231

Sir:

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

IN THE CLAIMS

Please cancel claims 65-69.

SEQUENCE LISTING

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

IN THE SPECIFICATION

The paragraph beginning page 4, line 19.

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

The paragraph beginning page 5, line 10.

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

C2

1

The paragraph beginning page 5, line 17.

Fig. 5. Dig-PCR of DNA from a stool sample. The 384 wells used in the experiment are displayed. Those colored blue contained 25 genome equivalents of DNA from normal cells. Each of these registered positive with MB-RED and the RED/GREEN ratios were 1.0 +/- 0.1 (mean +/- 1 standard deviation). The wells colored yellow contained no template DNA and each was negative with MB-RED (i.e., fluorescence <3500 fluorescence units.). The other wells contained diluted DNA from the stool sample. Those registering as positive with MB-RED were colored either red or green, depending on their RED/GREEN ratios. Those registering negative with MB-RED were colored white. PCR products from the indicated wells were used for automated sequence analysis. The sequence of WT *c-Ki-Ras* in well K1 (SEQ ID NO: 7), and mutant *c-Ki-Ras* in wells C10, E11, M10, and L12 (SEQ ID NO: 14), and well F21 (SEQ ID NO: 15) were analyzed.

C3

REMARKS

The Amendments

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

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

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

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

Respectfully submitted,

Date: February 20, 2002

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

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

MARKED UP VERSION TO SHOW CHANGES MADE

The paragraph beginning page 4, line 19.

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

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contained WT sequences. WT *c-Ki-Ras* (SEQ ID NO: 7), Gly12Asp (SEQ ID NO: 13), and Gly13Asp (SEQ ID NO: 9) were analyzed.

The paragraph beginning page 5, line 17.

Fig. 5. Dig-PCR of DNA from a stool sample. The 384 wells used in the experiment are displayed. Those colored blue contained 25 genome equivalents of DNA from normal cells. Each of these registered positive with MB-RED and the RED/GREEN ratios were 1.0 +/- 0.1 (mean +/- 1 standard deviation). The wells colored yellow contained no template DNA and each was negative with MB-RED (i.e., fluorescence <3500 fluorescence units.). The other wells contained diluted DNA from the stool sample. Those registering as positive with MB-RED were colored either red or green, depending on their RED/GREEN ratios. Those registering negative with MB-RED were colored white. PCR products from the indicated wells were used for automated sequence analysis. The sequence of WT *c-Ki-Ras* in well K1 (SEQ ID NO: 7), and mutant *c-Ki-Ras* in wells C10, E11, M10, and L12 (SEQ ID NO: 14), and well F21 (SEQ ID NO: 15) were analyzed.

SEQUENCE LISTING

<110> Vogelstein, Bert
Kinzler, Kenneth W.

<120> DIGITAL AMPLIFICATION

<130> 01107.00031

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

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

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Interview Summary	Application No.	Applicant(s)	
	09/613,826	VOGELSTEIN ET AL.	
	Examiner	Art Unit	
	Jeffrey Slew	1656	

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

(1) *Jeffrey Slew*. (3)____.

(2) *Michelle Holmes-Son*. (4)____.

Date of Interview: *04 March 2002* .

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

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

Claim(s) discussed: *1-64* .

Identification of prior art discussed: ____ .

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

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

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

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

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

Jeffrey Slew
3/4/02

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

_____
Examiner's signature, if required

#19

1637

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

Input Set : A:\107.31.ST25.txt
Output Set: N:\CRF3\03012002\I613826A.raw

3 <110> APPLICANT: Vogelstein, Bert
4 Kinzler, Kenneth W.
6 <120> TITLE OF INVENTION: DIGITAL AMPLIFICATION
8 <130> FILE REFERENCE: 01107.00031
10 <140> CURRENT APPLICATION NUMBER: 09/613,826A
11 <141> CURRENT FILING DATE: 2000-07-11
13 <150> PRIOR APPLICATION NUMBER: US 60/146,792
14 <151> PRIOR FILING DATE: 1999-08-02
16 <160> NUMBER OF SEQ ID NOS: 15
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20 <210> SEQ ID NO: 1
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RAW SEQUENCE LISTING DATE: 03/01/2002
PATENT APPLICATION: US/09/613,826A TIME: 15:29:37

Input Set : A:\107.31.ST25.txt
Output Set: N:\CRF3\03012002\I613826A.raw

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

Input Set : A:\107.31.ST25.txt
Output Set: N:\CRF3\03012002\I613826A.raw

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

Input Set : A:\107.31.ST25.txt
Output Set: N:\CRF3\03012002\I613826A.raw

File History Report

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

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

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

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

Additional comments:_____

Interview Summary	Application No.	Applicant(s)	
	09/613,829	TAKESHITA ET AL.	
	Examiner	Art Unit	
	Jeffrey Siew	1656	

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

(1) *Jeffrey Siew*. (3)_____.

(2) *Michelle Holmes Son*. (4)_____.

Date of Interview: *19 March 2002* .

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

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

Claim(s) discussed: *1-64* .

Identification of prior art discussed: _____ .

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

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

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

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

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

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

_____
Examiner's signature, if required

Notice of Allowability	Application No.	Applicant(s)	
	09/613,829	TAKESHITA ET AL.	
	Examiner	Art Unit	
	Jeffrey Siew	1656	

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

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

1. ☒ This communication is responsive to *Int 3/19/02*.
2. ☒ The allowed claim(s) is/are *1-64*.
3. ☒ The drawings filed on *11 July 2000* are accepted by the Examiner.
4. ☐ Acknowledgment is made of a claim for foreign priority under 35 U.S.C. § 119(a)-(d) or (f).
   a) ☐ All b) ☐ Some* c) ☐ None of the:
      1. ☐ Certified copies of the priority documents have been received.
      2. ☐ Certified copies of the priority documents have been received in Application No. _____ .
      3. ☐ Copies of the certified copies of the priority documents have been received in this national stage application from the International Bureau (PCT Rule 17.2(a)).

   * Certified copies not received: _____.
5. ☒ Acknowledgment is made of a claim for domestic priority under 35 U.S.C. § 119(e) (to a provisional application).
   (a) ☐ The translation of the foreign language provisional application has been received.
6. ☐ Acknowledgment is made of a claim for domestic priority under 35 U.S.C. §§ 120 and/or 121.

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

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

8. ☒ CORRECTED DRAWINGS must be submitted.
   (a) ☒ including changes required by the Notice of Draftsperson's Patent Drawing Review (PTO-948) attached
      1) ☒ hereto or 2) ☐ to Paper No. _____.
   (b) ☐ including changes required by the proposed drawing correction filed _____, which has been approved by the Examiner.
   (c) ☐ including changes required by the attached Examiner's Amendment / Comment or in the Office action of Paper No. _____.

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

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

Attachment(s)

1☐ Notice of References Cited (PTO-892)
3☒ Notice of Draftperson's Patent Drawing Review (PTO-948)
5☐ Information Disclosure Statements (PTO-1449), Paper No. _____.
7☐ Examiner's Comment Regarding Requirement for Deposit of Biological Material
2☐ Notice of Informal Patent Application (PTO-152)
4☒ Interview Summary (PTO-413), Paper No. 18-20
6☐ Examiner's Amendment/Comment
8☒ Examiner's Statement of Reasons for Allowance
9☐ Other

REASONS FOR ALLOWANCE

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

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

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

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

CONCLUSION

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

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

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

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

Jeffrey Siew

March 19, 2002

UNITED STATES PATENT AND TRADEMARK OFFICE

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

NOTICE OF ALLOWANCE AND FEE(S) DUE

22907 7590 03/24/2002

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

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

DATE MAILED: 03/24/2002

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

TITLE OF INVENTION: DIGITAL AMPLIFICATION

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

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

THE ISSUE FEE AND PUBLICATION FEE (IF REQUIRED) MUST BE PAID WITHIN THREE MONTHS FROM THE MAILING DATE OF THIS NOTICE OR THIS APPLICATION SHALL BE REGARDED AS ABANDONED. THIS STATUTORY PERIOD CANNOT BE EXTENDED. SEE 35 U.S.C. 151. THE ISSUE FEE DUE INDICATED ABOVE REFLECTS A CREDIT FOR ANY PREVIOUSLY PAID ISSUE FEE APPLIED IN THIS APPLICATION. THE PTOL-85B (OR AN EQUIVALENT) MUST BE RETURNED WITHIN THIS PERIOD EVEN IF NO FEE IS DUE OR THE APPLICATION WILL BE REGARDED AS ABANDONED.

HOW TO REPLY TO THIS NOTICE:

I. Review the SMALL ENTITY status shown above. If the SMALL ENTITY is shown as YES, verify your current SMALL ENTITY status:

A. If the status is changed, pay the PUBLICATION FEE (if required) and twice the amount of the ISSUE FEE shown above and notify the United States Patent and Trademark Office of the change in status, or

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

If the SMALL ENTITY is shown as NO:

A. Pay TOTAL FEE(S) DUE shown above, or

B. If applicant claimed SMALL ENTITY status before, or is now claiming SMALL ENTITY status, check the box below and enclose the PUBLICATION FEE and 1/2 the ISSUE FEE shown above.

☐ Applicant claims SMALL ENTITY status. See 37 CFR 1.27.

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

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

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

PTOL-85 (REV. 07-01) Approved for use through 01/31/2004.

PART B - FEE(S) TRANSMITTAL

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

MAILING INSTRUCTIONS. This form should be used for transmitting the ISSUE FEE and PUBLICATION FEE (if required). Blocks 1 through 4 should be complet where appropriate. All further correspondence including the Patent, advance orders and notification of maintenance fees will be mailed to the current correspondence address indicated unless corrected below or directed otherwise in Block 1, by (a) specifying a new correspondence address; and/or (b) indicating a separate "FEE ADDRESS" f maintenance fee notifications.

CURRENT CORRESPONDENCE ADDRESS (Note: Legibly mark-up with any corrections or use Block 1)

22907 7590 03/24/2002

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

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

Certificate of Mailing

I hereby certify that this Fee(s) Transmittal is being deposited with the United States Postal Service with sufficient postage for first class mail in an envelope addressed to the Box Issue Fee address above on the date indicated below.

(Depositor's nam
(Signatur
(Dat

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

TITLE OF INVENTION: DIGITAL AMPLIFICATION

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

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

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

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

☐ "Fee Address" indication (or "Fee Address" Indication form PTO/SB/47) attached

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

1 _____
2 _____
3 _____

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

PLEASE NOTE: Unless an assignee is identified below, no assignee data will appear on the patent. Inclusion of assignee data is only appropriate when an assignment has been previously submitted to the USPTO or is being submitted under separate cover. Completion of this form is NOT a substitute for filing an assignment.

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

Please check the appropriate assignee category or categories (will not be printed on the patent) ☐ individual ☐ corporation or other private group entity ☐ government

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

☐ Issue Fee
☐ Publication Fee
☐ Advance Order - # of Copies _____

4b. Payment of Fee(s):

☐ A check in the amount of the fee(s) is enclosed.
☐ Payment by credit card. Form PTO-2038 is attached.
☐ The Commissioner is hereby authorized by charge the required fee(s), or credit any overpayment, to Deposit Account Number _____ (enclose an extra copy of this form).

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

(Authorized Signature) (Date)

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

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

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

TRANSMIT THIS FORM WITH FEE(S)

UNITED STATES PATENT AND TRADEMARK OFFICE

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

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

22907 7590 03/24/2002

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

EXAMINER	
SIEW, JEFFREY	

ART UNIT	PAPER NUMBER
1637	21

DATE MAILED: 03/24/2002

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

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

If a continued prosecution application (CPA) was filed in the above-identified application, the filing date that determines patent term adjustment is the filing date of the most recent CPA.

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

Supplemental Notice of Allowability	Application No. 09/613,829	Applicant(s) TAKESHITA ET AL.	
	Examiner Jeffrey Siew	Art Unit 1656	

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

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

1. ☒ This communication is responsive to *Int 3/19/02*.
2. ☒ The allowed claim(s) is/are *1-64*.
3. ☒ The drawings filed on *11 July 2000* are accepted by the Examiner.
4. ☐ Acknowledgment is made of a claim for foreign priority under 35 U.S.C. § 119(a)-(d) or (f).
   a) ☐ All b) ☐ Some* c) ☐ None of the:
      1. ☐ Certified copies of the priority documents have been received.
      2. ☐ Certified copies of the priority documents have been received in Application No. _____ .
      3. ☐ Copies of the certified copies of the priority documents have been received in this national stage application from the International Bureau (PCT Rule 17.2(a)).

   * Certified copies not received: _____.
5. ☒ Acknowledgment is made of a claim for domestic priority under 35 U.S.C. § 119(e) (to a provisional application).
   (a) ☐ The translation of the foreign language provisional application has been received.
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7. ☐ A SUBSTITUTE OATH OR DECLARATION must be submitted. Note the attached EXAMINER'S AMENDMENT or NOTICE OF INFORMAL PATENT APPLICATION (PTO-152) which gives reason(s) why the oath or declaration is deficient.

8. ☒ CORRECTED DRAWINGS must be submitted.
   (a) ☒ including changes required by the Notice of Draftsperson's Patent Drawing Review (PTO-948) attached
      1) ☐ hereto or 2) ☒ to Paper No. 21.
   (b) ☐ including changes required by the proposed drawing correction filed _____, which has been approved by the Examiner.
   (c) ☐ including changes required by the attached Examiner's Amendment / Comment or in the Office action of Paper No. _____.

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

Attachment(s)

1☐ Notice of References Cited (PTO-892)
2☐ Notice of Informal Patent Application (PTO-152)
3☒ Notice of Draftperson's Patent Drawing Review (PTO-948)
4☒ Interview Summary (PTO-413), Paper No. 18 & 20
5☐ Information Disclosure Statements (PTO-1449), Paper No. _____.
6☐ Examiner's Amendment/Comment
7☐ Examiner's Comment Regarding Requirement for Deposit of Biological Material
8☒ Examiner's Statement of Reasons for Allowance
9☐ Other

REASONS FOR ALLOWANCE

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

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

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CONCLUSION

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

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

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

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

Jeffrey Siew

March 19, 2002

File History Report

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

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

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

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

Additional comments:_____

* Corrected Copy *

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UNITED STATES DEPARTMENT OF COMMERCE
United States Patent and Trademark Office
Address: COMMISSIONER OF PATENTS AND TRADEMARKS
Washington, D.C. 20231
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NOTICE OF ALLOWANCE AND FEE(S) DUE

22907 7590 03/26/2002

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

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

DATE MAILED: 03/26/2002

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

TITLE OF INVENTION, DIGITAL AMPLIFICATION

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

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

THE ISSUE FEE AND PUBLICATION FEE (IF REQUIRED) MUST BE PAID WITHIN THREE MONTHS FROM THE MAILING DATE OF THIS NOTICE OR THIS APPLICATION SHALL BE REGARDED AS ABANDONED. THIS STATUTORY PERIOD CANNOT BE EXTENDED. SEE 35 U.S.C. 151. THE ISSUE FEE DUE INDICATED ABOVE REFLECTS A CREDIT FOR ANY PREVIOUSLY PAID ISSUE FEE APPLIED IN THIS APPLICATION. THE PTOL-85B (OR AN EQUIVALENT) MUST BE RETURNED WITHIN THIS PERIOD EVEN IF NO FEE IS DUE OR THE APPLICATION WILL BE REGARDED AS ABANDONED.

HOW TO REPLY TO THIS NOTICE:

I. Review the SMALL ENTITY status shown above. If the SMALL ENTITY is shown as YES, verify your current SMALL ENTITY status:

A. If the status is changed, pay the PUBLICATION FEE (if required) and twice the amount of the ISSUE FEE shown above and notify the United States Patent and Trademark Office of the change in status, or

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

If the SMALL ENTITY is shown as NO:

A. Pay TOTAL FEE(S) DUE shown above, or

B. If applicant claimed SMALL ENTITY status before, or is now claiming SMALL ENTITY status, check the box below and enclose the PUBLICATION FEE and 1/2 the ISSUE FEE shown above.

☐ Applicant claims SMALL ENTITY status. See 37 CFR 1.27.

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

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

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

PTOL-85 (REV. 07-01) Approved for use through 01/31/2004.

PART B - FEE(S) TRANSMITTAL

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

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22907 7590 03/26/2002

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SUITE 1100
WASHINGTON, DC 20001

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(Signature)
(Date)

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

TITLE OF INVENTION: DIGITAL AMPLIFICATION

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

EXAMINER	ART UNIT	CLASS-SUBCLASS
SIBW, JEFFREY	1637	435-006000

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

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

☐ "Fee Address" indication (or "Fee Address" Indication form PTO/SB/47) attached.

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

1 _____
2 _____
3 _____

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

PLEASE NOTE: Unless an assignee is identified below, no assignee data will appear on the patent. Inclusion of assignee data is only appropriate when an assignment has been previously submitted to the USPTO or is being submitted under separate cover. Completion of this form is NOT a substitute for filing an assignment.

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

Please check the appropriate assignee category or categories (will not be printed on the patent) ☐ individual ☐ corporation or other private group entity ☐ government

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

☐ Issue Fee
☐ Publication Fee
☐ Advance Order - # of Copies _____

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☐ A check in the amount of the fee(s) is enclosed.
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Supplemental

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

22907 7590 03/26/2002

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

EXAMINER	
SIEW, JEFFREY	

ART UNIT	PAPER NUMBER
1637	22

DATE MAILED: 03/26/2002

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

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

If a continued prosecution application (CPA) was filed in the above-identified application, the filing date that determines patent term adjustment is the filing date of the most recent CPA.

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

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PATENT & TRADEMARK

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

APPLICATION NO.	FILING DATE	FIRST NAMED INVENTOR	ATTORNEY DOCKET NO.	CONFIRMATION NO.
09/613,826	07/11/2000	Bert Vogelstein	01107.00051	9893

TITLE OF INVENTION: DIGITAL AMPLIFICATION

TOTAL CLAIMS	APPLN. TYPE	SMALL ENTITY	ISSUE FEE	PUBLICATION FEE	TOTAL FEE(S) DUE	DATE DUE
64	nonprovisional	YES	$640	$0	$640	08/26/2002

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

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

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

☐ "Fee Address" indication (or "Fee Address" Indication form PTO/SB/47) attached.

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

1 _____
2 Banner & Witcoff, Ltd.
3 _____

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

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(A) NAME OF ASSIGNEE: Johns Hopkins University

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

Please check the appropriate assignee category or categories (will not be printed on the patent) ☐ individual ☒ corporation or other private group entity ☐ government

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

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(Authorized Signature) _____ (Date)

Michelle Holmes-Sun, Reg. No. 47,660 May 21, 2002

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05/22/2002 TBESHIR 00000060 190733 09613826

01 FC:242 640.00 CH
02 FC:1561 30.00 CH

TRANSMIT THIS FORM WITH FEE(S)

PATENT APPLICATION

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

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

INFORMATION DISCLOSURE STATEMENT

Assistant Commissioner for Patents
Washington, D.C. 20231

Sir:

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

Each item of information contained in the information disclosure statement was first cited in any communication from a foreign patent office in a counterpart foreign application not more than three months prior to the filing of the information disclosure statement.

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

Respectfully submitted,

Date: January 23, 2002

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

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

PTO/SB/08A (10-01)
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Substitute for form 1449A/PTO

INFORMATION DISCLOSURE STATEMENT BY APPLICANT

(use as many sheets as necessary)

Sheet 1 of 2

Complete If Known	
Application Number	09/613,826
Filing Date	July 11, 2000
First Named Inventor	Bert Vogelstein et al.
Group Art Unit	1656
Examiner Name	J. Siew
Attorney Docket Number	01107.00031

U.S. PATENT DOCUMENTS

Examiner Initials*	Cite No.[1]	Document Number: Number - [Kind Code][2] (if known)	Publication Date MM-DD-YYYY	Name of Patentee or Applicant of Cited Document	Pages, Columns, Lines, Where Relevant Passages or Relevant Figures Appear
JS		US-5,804,383	09-08-1998	Gruenert et al.	
JS		US-5,859,663	01-12-1999	Nissen et al.	
		US-			
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		US-			
		US-			

FOREIGN PATENT DOCUMENTS

Examiner Initials*	Cite No.[1]	Foreign Patent Document: Country Code[3] - Number[4] - Kind Code[5] (if known)	Publication Date MM-DD-YYYY	Name of Patentee or Applicant of Cited Document	Pages, Columns, Lines, Where Relevant Passages or Relevant Figures Appear	T[6]
JS		WO 95/13399	05-18-1995			
JS		EP 0643140 A	03-15-1995			
JS		WO 99/13113	03-18-1999			

Examiner Signature		Date Considered	4/15/02

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

[1] Applicant's unique citation designation number (optional). [2] See Kinds Codes of USPTO Patent Documents at www.uspto.gov or MPEP 901.04. [3] Enter Office that issued the document, by the two-letter code (WIPO Standard ST.3). [4] For Japanese patent documents, the indication of the year of the reign of the Emperor must precede the serial number of the patent document. [5] Kind of document by the appropriate symbols as indicated on the document under WIPO Standard ST. 16 if possible. [6] Applicant is to place a check mark here if English language Translation is attached.

Burden Hour Statement: This form is estimated to take 2.0 hours to complete. Time will vary depending upon the needs of the individual case. Any comments on the amount of time you are required to complete this form should be sent to the Chief Information Officer, U.S. Patent and Trademark Office, Washington, DC 20231. DO NOT SEND FEES OR COMPLETED FORMS TO THIS ADDRESS. SEND TO: Assistant Commissioner for Patents, Washington, DC 20231.

PTO/SB/08B(10-01)
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Substitute for form 1449/PTO

INFORMATION DISCLOSURE STATEMENT BY APPLICANT

(use as many sheets as necessary)

Sheet 2 of 2

Complete If Known	
Application Number	09/613,826
Filing Date	July 11, 2000
First Named Inventor	Bert Vogelstein et al.
Group Art Unit	1656
Examiner Name	J. Siew
Attorney Docket Number	01107.00031

OTHER PRIOR ART – NON PATENT LITERATURE DOCUMENTS

Examiner Initials *	Cite No.[1]	Include name of the author (in CAPITAL LETTERS), title of the article (when appropriate), title of the item (book, magazine, journal, serial, symposium, catalog, etc.), date, page(s), volume-issue number(s), publisher, city and/or country where published.	T[2]
SS		A. PIATEK et al., "Molecular Beacon Sequence Analysis for Detecting Drug Resistance in Mycobacterium Tuberculosis", Nature Biotechnology, April 1998, pp. 359-363, Vol. 16, No. 4	
		S. TYAGI et al., "Multicolor Molecular Beacons for allele discrimination", Nature Biotechnology, pp. 303-308, January 1998, Vol. 16, No. 1	
		J. A.M. VET et al., "Multiplex Detection of Four Pathogenic Retroviruses Using Molecular Beacons", Proceedings of the National Academy of Science of the United States", May 25, 1999, pp. 6394-6399, Vol. 96, No. 11	
		S. TYAGI et al., "Molecular Beacons: probes that Fluoresce Upon Hybridization", Nature Biotechnology, 1996, pp. 303-308, Vol. 14, No. 3	
		W. P. HALFORD et al., "The Inherent Quantitative Capacity of the Reverse Transcription-Polymerase Chain Reaction", Analytical Biochemistry, January 15, 1999, pp. 101-191, Vol. 268, No. 2	
		B. VOGELSTEIN et al., "Digital PCR", Proceedings of the National Academy of Sciences of the United States, August 3, 1999, pp. 9236-9241, Vol. 96, No. 16	
SS		K. D.E. EVERETT et al, "Identification of nine species of the Chlamydiaceae Using PCR-RFLP", April 1999, pp. 903-913, Vol. 49, No. 2	

Examiner Signature		Date Considered	4/15/02

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

[1] Unique citation designation number (optional). [2] Applicant is to place a check mark here if English language Translation is attached.

Burden Hour Statement: This form is estimated to take 2.0 hours to complete. Time will vary depending upon the needs of the individual case. Any comments on the amount of time you are required to complete this form should be sent to the Chief Information Officer, U.S. Patent and Trademark Office, Washington, DC 20231. DO NOT SEND FEES OR COMPLETED FORMS TO THIS ADDRESS. SEND TO: Assistant Commissioner for Patents, Washington, DC 20231.

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U.S. Patent and Trademark Office: U.S. DEPARTMENT OF COMMERCE
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FEE TRANSMITTAL for FY 2002

Patent fees are subject to annual revision.

TOTAL AMOUNT OF PAYMENT ($) 180

Complete if Known	
Application Number	09/613,626
Filing Date	July 11, 2000
First Named Inventor	Bert Vogelstein et al.
Examiner Name	J. Siew
Group / Art Unit	1656
Attorney Docket No.	01107.00031

METHOD OF PAYMENT (check all that apply)

☐ Check ☐ Credit card ☐ Money Order ☐ Other ☐ None

☒ Deposit Account:

Deposit Account Number: Banner & Witcoff, Ltd.

Deposit Account Name: 19-0733

The Commissioner is authorized to: *(check all that apply)*
☒ Charge fee(s) indicated below ☒ Credit any overpayments
☒ Charge any additional fee(s) during the pendency of this application
☐ Charge fee(s) indicated below, except for the filing fee to the above-identified deposit account.

FEE CALCULATION

1. BASIC FILING FEE

Large Entity Fee Code	Large Entity Fee ($)	Small Entity Fee Code	Small Entity Fee ($)	Fee Description	Fee Paid
101	740	201	370	Utility filing fee	
106	330	206	165	Design filing fee	
107	510	207	255	Plant filing fee	
108	740	208	370	Reissue filing fee	
114	160	214	80	Provisional filing fee	
		SUBTOTAL (1)			($) 0

2. EXTRA CLAIM FEES

		Extra Claims		Fee from below		Fee Paid
Total Claims	-20 **	0	X		=	0
Independent Claims	-3 **	0	X		=	0
Multiple Dependent			X		=	0

Large Entity Fee Code	Large Entity Fee ($)	Small Entity Fee Code	Small Entity Fee ($)	Fee Description
103	18	203	9	Claims in excess of 20
102	84	202	42	Independent claims in excess of 3
104	280	204	140	Multiple dependent claim, if not paid
109	84	209	42	** Reissue independent claims over original patent
110	18	210	9	** Reissue claims in excess of 20 and over original patent
		SUBTOTAL (2)		($) 0

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

FEE CALCULATION (continued)

3. ADDITIONAL FEES

Large Entity Fee Code	Large Entity Fee ($)	Small Entity Fee Code	Small Entity Fee ($)	Fee Description	Fee Paid
105	130	205	65	Surcharge - late filing fee or oath	
127	50	227	25	Surcharge - late provisional filing fee or cover sheet.	
139	130	139	130	Non-English specification	
147	2,520	147	2,520	For filing a request for reexamination	
112	920*	112	920*	Requesting publication of SIR prior to Examiner action	
113	1,840*	113	1,840*	Requesting publication of SIR after Examiner action	
115	110	215	55	Extension for reply within first month	
116	400	216	200	Extension for reply within second month	
117	920	217	460	Extension for reply within third month	
118	1,440	218	720	Extension for reply within fourth month	
128	1,960	228	980	Extension for reply within fifth month	
119	320	219	160	Notice of Appeal	
120	320	220	160	Filing a brief in support of an appeal	
121	280	221	140	Request for oral hearing	
138	1,510	138	1,510	Petition to institute a public use proceeding	
140	110	240	55	Petition to revive – unavoidable	
141	1,280	241	640	Petition to revive – unintentional	
142	1,280	242	640	Utility issue fee (or reissue)	
143	460	243	230	Design issue fee	
144	620	244	310	Plant issue fee	
122	130	122	130	Petitions to the Commissioner	
123	50	123	50	Processing fee under 37 CFR 1.17 (q)	
126	180	126	180	Submission of Information Disclosure Stmt	180
581	40	581	40	Recording each patent assignment per property (times number of properties)	
146	740	246	370	Filing a submission after final rejection (37 CFR § 1.129(a))	
149	740	249	370	For each additional invention to be examined (37 CFR § 1.129(b))	
179	740	279	370	Request for Continued Examination (RCE)	
169	900	169	900	Request for expedited examination of a design application	
				Other fee (specify)	

*Reduced by Basic Filing Fee Paid SUBTOTAL (3) ($) 180

SUBMITTED BY				Complete (if applicable)	
Name (Print/Type)	Michelle L. Holmes-Sloan	Registration No. Attorney/Agent	47,630	Telephone	202-508-9100
Signature				Date	January 23, 2002

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Total Number of Pages in This Submission

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

ENCLOSURES *(check all that apply)*

- [X] Fee Transmittal Form
  - [ ] Fee Attached
- [ ] Amendment / Response
  - [ ] After Final
  - [ ] Affidavits/declaration(s)
- [ ] Extension of Time Request
- [ ] Express Abandonment Request
- [X] Information Disclosure Statement
- [ ] Certified Copy of Priority Document(s)
- [ ] Response to Missing Parts/ Incomplete Application
  - [ ] Response to Missing Parts under 37 CFR 1.52 or 1.53
- [ ] Assignment Papers *(for an Application)*
- [ ] Drawing(s)
- [ ] Licensing-related Papers
- [ ] Petition
- [ ] Petition to Convert to a Provisional Application
- [ ] Power of Attorney, Revocation Change of Correspondence Address
- [ ] Terminal Disclaimer
- [ ] Request for Refund
- [ ] CD, Number of CD(s) ____
- [ ] After Allowance Communication to Group
- [ ] Appeal Communication to Board of Appeals and Interferences
- [ ] Appeal Communication to Group *(Appeal Notice, Brief, Reply Brief)*
- [ ] Proprietary Information
- [ ] Status Letter
- [X] Other Enclosure(s) *(please identify below)*: PTO-1449 (w/references)

Remarks

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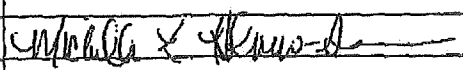
Signature	
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FAX: 202.508.9299
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FACSIMILE TRANSMITTAL SHEET

TO:	FROM:
Jeffrey Slew	Michelle L. Holmes-Son
COMPANY:	DATE:
United States Patent and Trademark Office	April 15, 2002
FAX NUMBER:	TOTAL NO. OF PAGES:
(703)-746-5178	7, including this cover sheet
RE:	OUR REFERENCE NO.:
U.S. Serial Number: 09/613,826	01107.00031

If you do not receive all page(s) or have any problems receiving this transmission, please call:

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

COMMENTS:

Pursuant to our telephone conversation of this morning, attached please find a copy of our Information Disclosure Statement filed January 29, 2002. Please return by fax after you are satisfied with your review of the document.

Yours truly,

Michelle L. Holmes-Son (47,660)

#23 Formal Drwgs

PATENT

6/17/02

#25 - Issue

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

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

For: DIGITAL AMPLIFICATION

SUBMISSION OF FORMAL DRAWINGS

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Washington, D.C. 20231
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Dear Sir:

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

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

Respectfully submitted,

Date: May 21, 2002

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

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

03/02

FIG. 1A

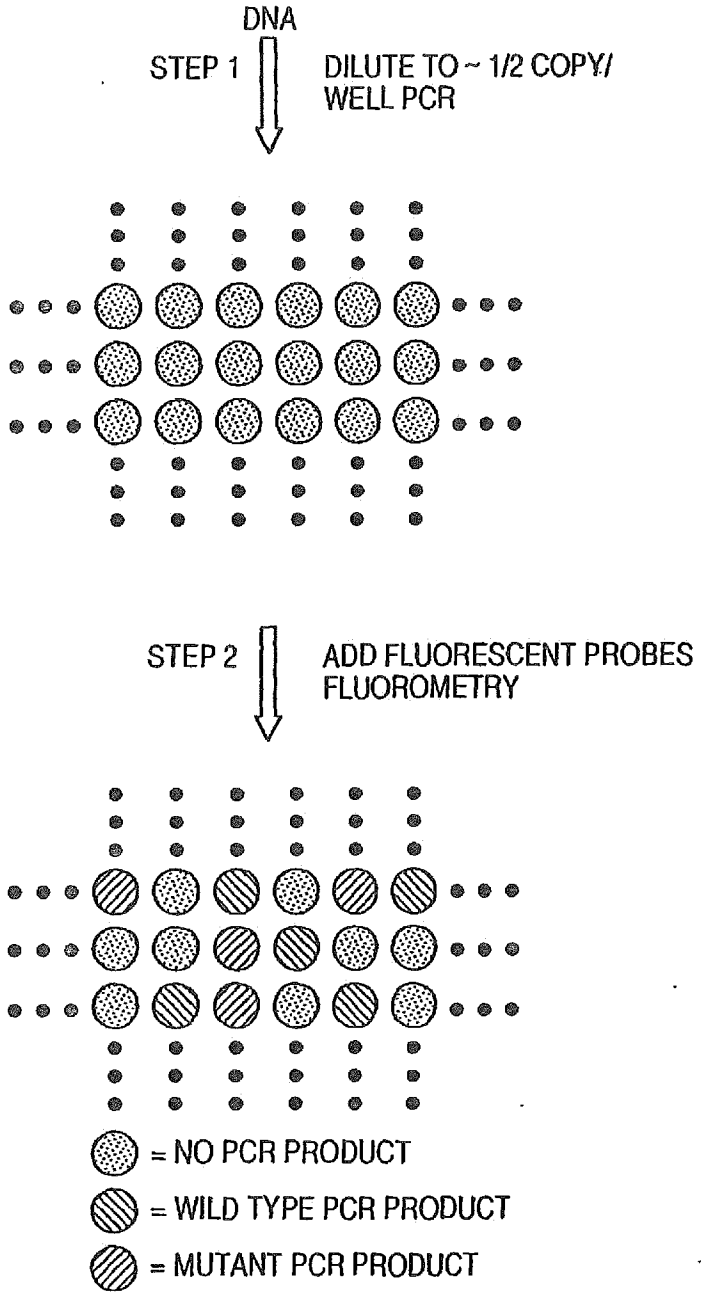
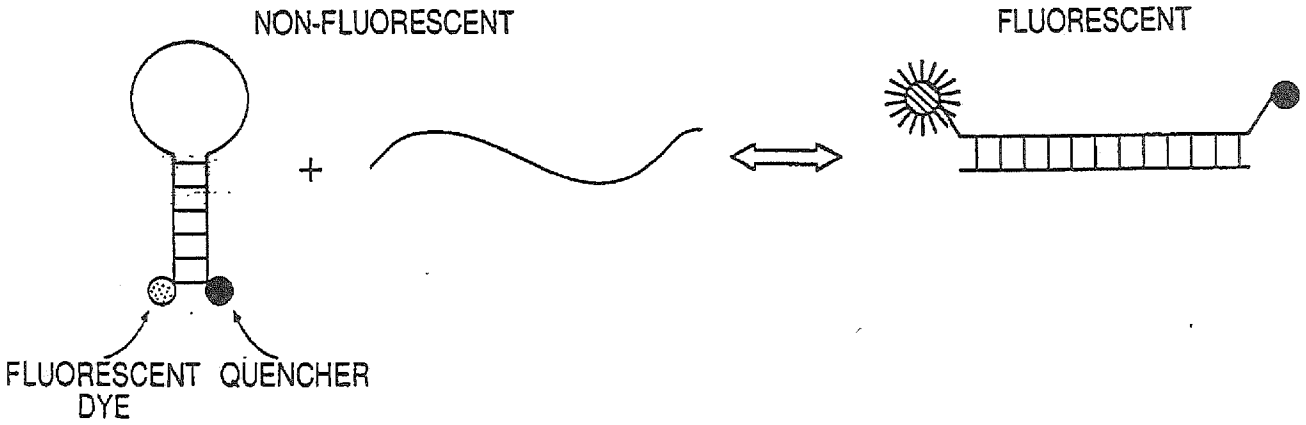

DNA
STEP 1
DILUTE TO ~ 1/2 COPY/ WELL PCR
STEP 2
ADD FLUORESCENT PROBES FLUOROMETRY
= NO PCR PRODUCT
= WILD TYPE PCR PRODUCT
= MUTANT PCR PRODUCT

FIG. 1B



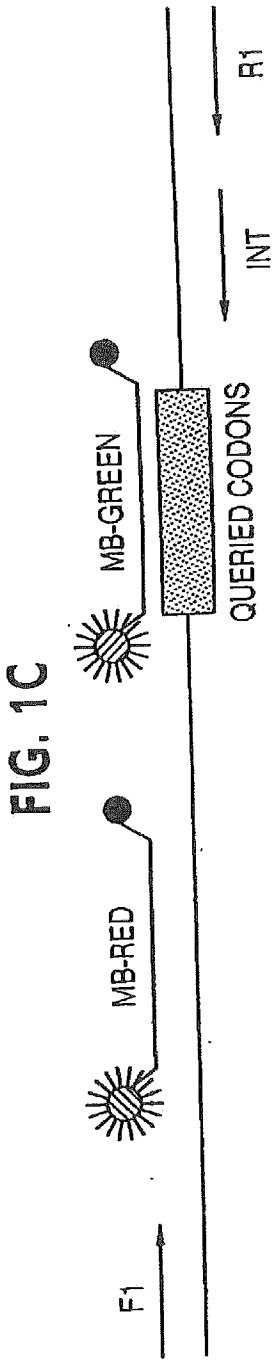
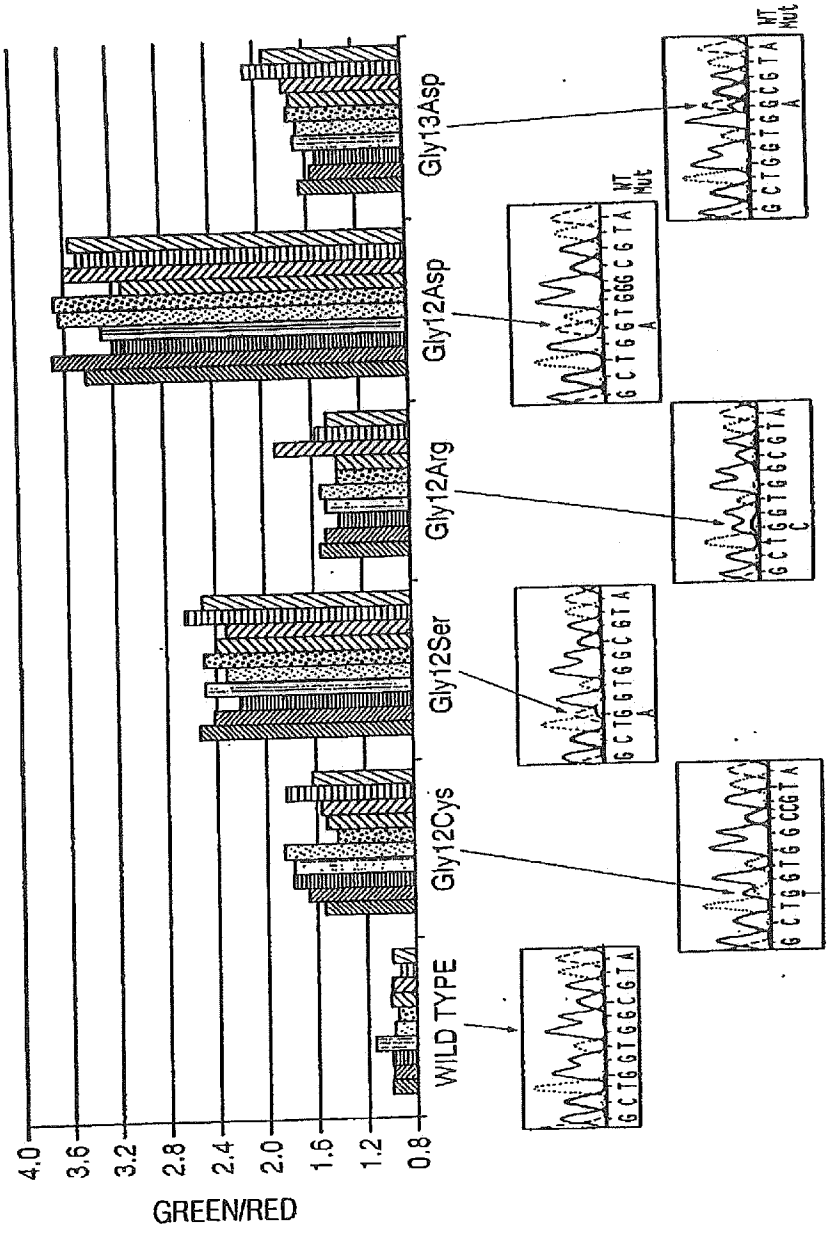


FIG. 1C

FIG. 2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3.6
3.2
2.8
2.4
2.0
1.6
1.2
0.8
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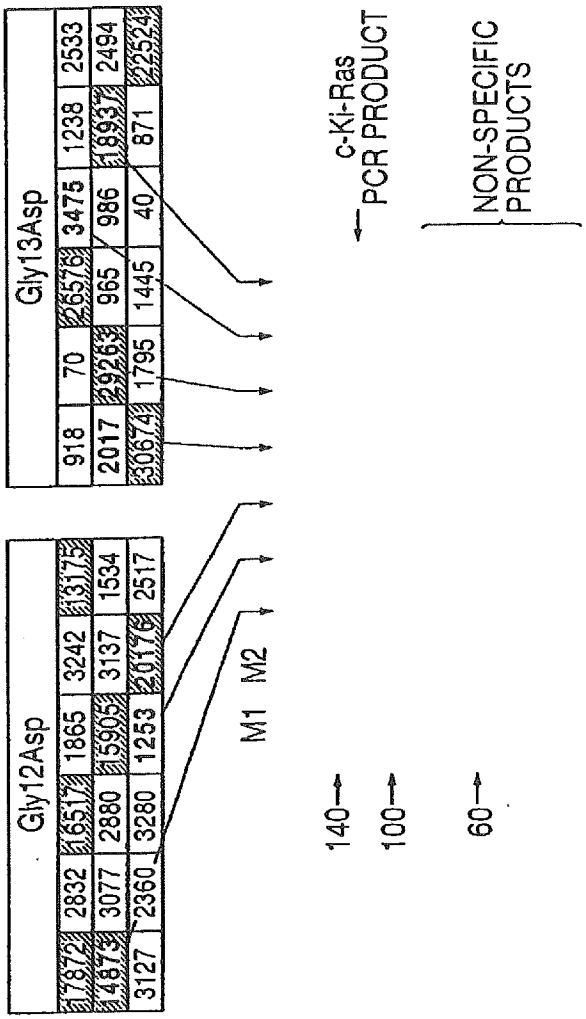
Gly12Ser
Gly12Arg
Gly12Asp
Gly13Asp
WT
Mut

FIG. 3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1651
1865
3242
3175
4873
3077
2880
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1534
3127
2360
3280
1253
2176
2517
Gly13Asp
918
70
2657
3475
1238
2533
2017
2926
965
986
1893
2494
3067
1795
1445
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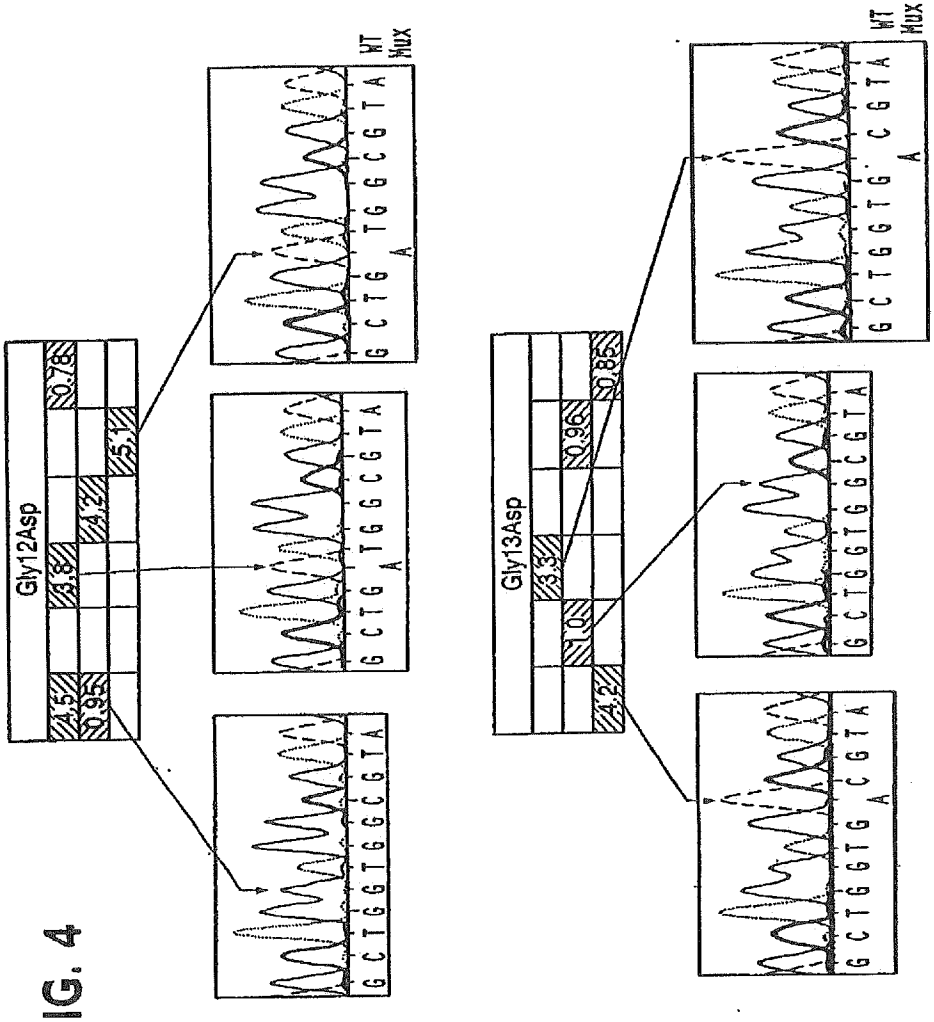
871
2252
M1
M2
c-Ki-Ras
PCR PRODUCT
NON-SPECIFIC
PRODUCTS
140
100
60
20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3.8
4.2
5.1
0.78
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G C T G A T G G C G T A
WT
Mux
Gly13Asp
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1.0
3.3
0.96
0.85
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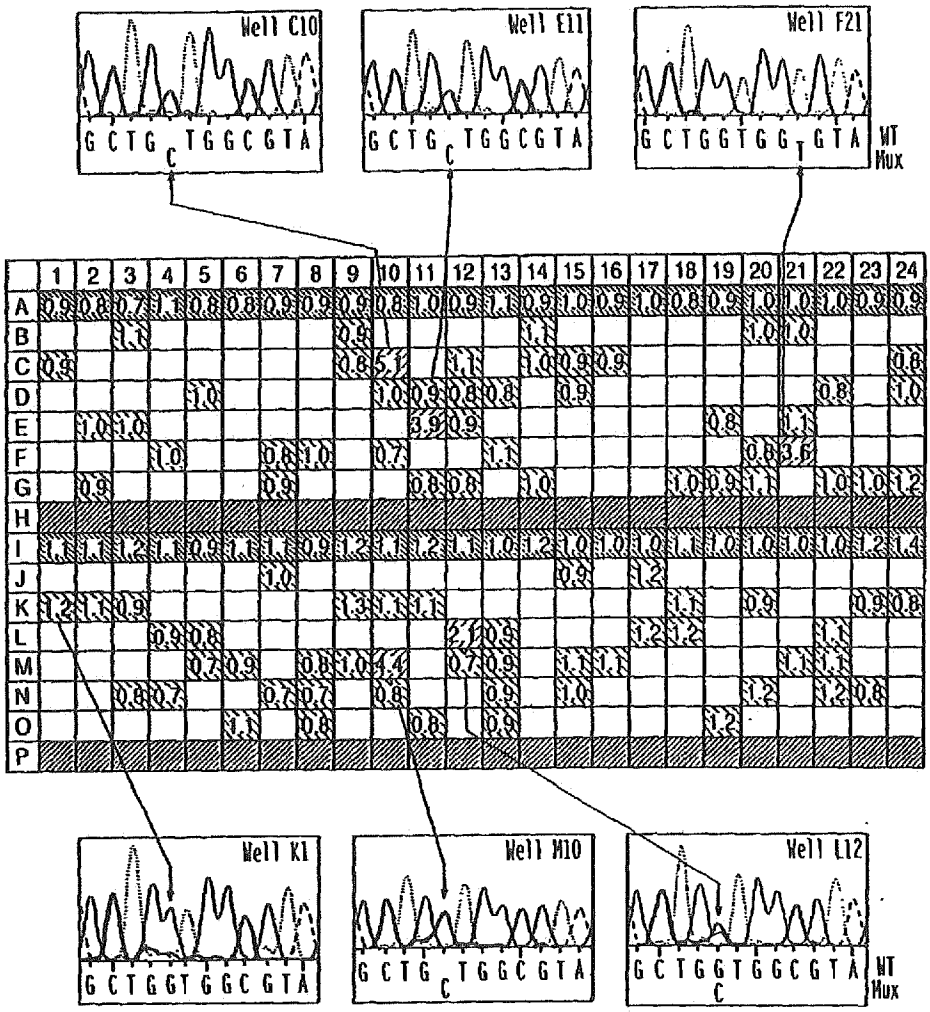
G C T G G T G G C G T A
G C T G G T G A C G T A
WT
Mux

FIG. 4

FIG. 5


Well C10
G C T G C T G G C G T A
Well E11
G C T G C T G G C G T A
Well F21
G C T G G T G G T G T A
WT Mux
Well K1
G C T G G T G G C G T A
Well M10
G C T G C T G G C G T A
Well L12
G C T G G T G G C G T A
WT Mux

☒ PATENT ☐ DESIGN B&W Ref. 01107.00021 Date May 21, 2002

☐ HAND CARRY Group/Section _____ Bldg _____ Rm _____

Serial/Patent No. 09/613,826 Atty/Sec SAK/MHS/sel

Inventor Bert Vogelstein et al Client JHU

Title Digital Amplification

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The following has been received in the U.S. Patent and Trademark Office on the date stamped hereon:

☐ _____ total pp Spec., including : # of Claims _____ (# of independent claims _____). ☐ Abstract

☒ Drawings : ☒ Formal ☐ Informal 1A, 1B, 1C, 2, 3, # of distinct sheets 7 : Figs. 4, 5

☐ Declaration/Power of Attorney : ☐ Executed ☐ Unexecuted

☐ Assignment w/PTO Cover Sheet

☐ IDS w/PTO 1449 ☐ References ☐ w/Fee

☐ Preliminary Amendment

☐ Priority Claim (Foreign or U.S. Provisional. B&W # _____)

Country	Appl. #	Date
☐ w/Foreign Priority Document(s)		

☐ Application : ☐ CIP ☐ Continuation ☐ Divisional

Parent Ser. No. _____ B&W# _____

☐ U.S. Provisional _____ pp Spec/Claims; Cover Sheet

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☐ Petition for Extension of Time until _____

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☐ Request for Approval of Drawing Changes

☐ Notice of Appeal & Fee

☐ Brief : ☐ Appeal & Fee ☐ Reply

☐ Request for Oral Hearing

☒ Issue Fee ☒ Advance Patent Copies (# ordered 10) Notice of Allowance dtd 2-26-02

☐ Amendment under 37 CFR 1.312

☐ Request for Certificate of Correction

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TRANSMITTAL FORM

(to be used for all correspondence after initial filing)

Application Number	09/613,826
Filing Date	July 11, 2000
First Named Inventor	Bert Vogelstein
Group Art Unit	1637
Examiner Name	Jeffrey Siew
Attorney Docket Number	001107.00031

Total Number of Pages in This Submission

ENCLOSURES *(check all that apply)*

- ☐ Fee Transmittal Form
  - ☐ Fee Attached
- ☐ Amendment / Response
  - ☐ After Final
  - ☐ Affidavits/declaration(s)
- ☐ Extension of Time Request
- ☐ Express Abandonment Request
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- ☐ Certified Copy of Priority Document(s)
- ☐ Response to Missing Parts/ Incomplete Application
  - ☐ Response to Missing Parts under 37 CFR 1.52 or 1.53
- ☐ Assignment Papers *(for an Application)*
- ☒ Drawing(s) 7 sheets (Figs. 1A, 1B, 1C, 2, 3, 4, and 5)
- ☐ Licensing-related Papers
- ☐ Petition
- ☐ Petition to Convert to a Provisional Application
- ☐ Power of Attorney, Revocation Change of Correspondence Address
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- ☐ 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
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Signature	Michelle Holmes-Son
Date	July 8, 2002

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-4-25-2002

PATENT

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

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

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

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- ☐ **PTO 948**
- ☐ **PTO 1474**
- ☐ **Assignment**
- ☐ **Cover page**

Additional comments:Paper #25 same as Paper #23

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Date: 15 Apr 2002

To: Michelle L Holmes-Son	**From:** Jeffrey Siew
Application/Control Number: 09/613,826	**Art Unit:** 1656
Fax No.: (202) 508-9299	**Phone No.:** 703-305-3886
Voice No.: (202) 508-9100	**Return Fax No.:** 703-308-4556
Re:	**CC:**

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Comments:
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JSiew

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Application/Control Number: 09/813,528	Art Unit: 1656
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Voice No.: (202) 508-9100	Return Fax No.: 703-308-4556
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Fax No.: (202) 508-9299	**Phone No.:** 703-305-3886
Voice No.: (202) 508-9100	**Return Fax No.:** 703-308-4556
Re:	**CC:**

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Jeffrey Siew

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Application No.: 09/613826

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

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

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

☒ 1. This application clearly fails to comply with the requirements of 37 C.F.R. 1.821-1.825. Applicant's attention is directed to the final rulemaking notice published at 55 FR 18230 (May 1, 1990), and 1114 OG 29 (May 15, 1990). If the effective filing date is on or after July 1, 1998, see the final rulemaking notice published at 63 FR 29620 (June 1, 1998) and 1211 OG 82 (June 23, 1998).

☒ 2. This application does not contain, as a separate part of the disclosure on paper copy, a "Sequence Listing" as required by 37 C.F.R. 1.821(c).

☒ 3. A copy of the "Sequence Listing" in computer readable form has not been submitted as required by 37 C.F.R. 1.821(e).

☐ 4. A copy of the "Sequence Listing" in computer readable form has been submitted. However, the content of the computer readable form does not comply with the requirements of 37 C.F.R. 1.822 and/or 1.823, as indicated on the attached copy of the marked -up "Raw Sequence Listing."

☐ 5. The computer readable form that has been filed with this application has been found to be damaged and/or unreadable as indicated on the attached CRF Diskette Problem Report. A Substitute computer readable form must be submitted as required by 37 C.F.R. 1.825(d).

☐ 6. The paper copy of the "Sequence Listing" is not the same as the computer readable from of the "Sequence Listing" as required by 37 C.F.R. 1.821(e).

☐ 7. Other: _____

Applicant Must Provide:

☒ An initial or substitute computer readable form (CRF) copy of the "Sequence Listing".

☒ An initial or substitute paper copy of the "Sequence Listing", as well as an amendment directing its entry into the specification.

☒ A statement that the content of the paper and computer readable copies are the same and, where applicable, include no new matter, as required by 37 C.F.R. 1.821(e) or 1.821(f) or 1.821(g) or 1.825(b) or 1.825(d).

For questions regarding compliance to these requirements, please contact:

For Rules Interpretation, call (703) 308-4216
For CRF Submission Help, call (703) 308-4212
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APPLICATION NUMBER: 08/613826

Total Fee Calculation

	Fee Code	Total # Claims		Number Extra	X	Fee Ca. Entity	Fee Sm. Entity	=	Total
Basic Filing Fee	101 103						690	=	
Total Claims >20	103 203	64	-20 =	44	X		22	=	
Independent Claims >1	102 202	5	-3 =	2	X		156	=	
Multi. Dep Claim Present	104 204						130	=	
Surcharge	105 205							=	
English Translation	139								
									1768

TOTAL FEE CALCULATION

Fees due upon filing the application is:

Total Filing Fees Due = $ 1768

Less Filing Fees Submitted - $ /

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Office of Initial Patent Examination

Figure 7

FORM OPE-RAM-01 (Rev. 12/97)

PATENT COOPERATION TREATY

From the INTERNATIONAL SEARCHING AUTHORITY 01107.00030

PCT

NOTIFICATION OF TRANSMITTAL OF THE INTERNATIONAL SEARCH REPORT OR THE DECLARATION

(PCT Rule 44.1)

DOCKETED
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Article 19 Amendment
21 FE 2002

To:
BANNER & WITCOFF, LTD.
Attn. KAGAN, Sarah A.
1001 G Street, N.W.
Eleventh Floor
Washington, DC 20001-4597
UNITED STATES OF AMERICA

Date of mailing (day/month/year)	21/12/2001
Applicant's or agent's file reference: 01107.00030	FOR FURTHER ACTION See paragraphs 1 and 4 below
International application No.: PCT/US 00/20740	International filing date (day/month/year): 31/07/2000

Applicant: THE JOHNS HOPKINS UNIVERSITY et al.

1. [X] The applicant is hereby notified that the International Search Report has been established and is transmitted herewith.

Filing of amendments and statement under Article 19:
The applicant is entitled, if he so wishes, to amend the claims of the International Application (see Rule 46):

When? The time limit for filing such amendments is normally 2 months from the date of transmittal of the International Search Report; however, for more details, see the notes on the accompanying sheet.

Where? Directly to the International Bureau of WIPO
34, chemin des Colombettes
1211 Geneva 20, Switzerland
Fascimile No.: (41-22) 740.14.35

For more detailed instructions, see the notes on the accompanying sheet.

2. [ ] The applicant is hereby notified that no International Search Report will be established and that the declaration under Article 17(2)(a) to that effect is transmitted herewith.

3. [ ] With regard to the protest against payment of (an) additional fee(s) under Rule 40.2, the applicant is notified that:

[ ] the protest together with the decision thereon has been transmitted to the International Bureau together with the applicant's request to forward the texts of both the protest and the decision thereon to the designated Offices.

[ ] no decision has been made yet on the protest; the applicant will be notified as soon as a decision is made.

4. **Further action(s):** The applicant is reminded of the following:

Shortly after **18 months** from the priority date, the international application will be published by the International Bureau. If the applicant wishes to avoid or postpone publication, a notice of withdrawal of the international application, or of the priority claim, must reach the International Bureau as provided in Rules 90*bis*.1 and 90*bis*.3, respectively, before the completion of the technical preparations for international publication.

Within **19 months** from the priority date, a demand for international preliminary examination must be filed if the applicant wishes to postpone the entry into the national phase until 30 months from the priority date (in some Offices even later).

Within **20 months** from the priority date, the applicant must perform the prescribed acts for entry into the national phase before all designated Offices which have not been elected in the demand or in a later election within 19 months from the priority date or could not be elected because they are not bound by Chapter II.

Name and mailing address of the International Searching Authority	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL-2280 HV Rijswijk Tel. (+31-70) 340-2040, Tx. 31 651 epo nl, Fax: (+31-70) 340-3016	Catherine Humbert

Form PCT/ISA/220 (July 1998)

NOTES TO FORM PCT/ISA/220

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

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

INSTRUCTIONS CONCERNING AMENDMENTS UNDER ARTICLE 19

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

What parts of the international application may be amended?

Under Article 19, only the claims may be amended.

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

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

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

Where not to file the amendments?

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

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

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

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

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

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

What documents must/may accompany the amendments?

Letter (Section 205(b)):

The amendments must be submitted with a letter.

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

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

NOTES TO FORM PCT/ISA/220 (continued)

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

(i) the claim is unchanged;

(ii) the claim is cancelled;

(iii) the claim is new;

(iv) the claim replaces one or more claims as filed;

(v) the claim is the result of the division of a claim as filed.

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

1. [Where originally there were 48 claims and after amendment of some claims there are 51]:
"Claims 1 to 29, 31, 32, 34, 35, 37 to 48 replaced by amended claims bearing the same numbers; claims 30, 33 and 36 unchanged; new claims 49 to 51 added."

2. [Where originally there were 15 claims and after amendment of all claims there are 11]:
"Claims 1 to 15 replaced by amended claims 1 to 11."

3. [Where originally there were 14 claims and the amendments consist in cancelling some claims and in adding new claims]:
"Claims 1 to 6 and 14 unchanged; claims 7 to 13 cancelled; new claims 15, 16 and 17 added." or
"Claims 7 to 13 cancelled; new claims 15, 16 and 17 added; all other claims unchanged."

4. [Where various kinds of amendments are made]:
"Claims 1-10 unchanged; claims 11 to 13, 18 and 19 cancelled; claims 14, 15 and 16 replaced by amended claim 14; claim 17 subdivided into amended claims 15, 16 and 17; new claims 20 and 21 added."

"Statement under article 19(1)" (Rule 46.4)

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

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

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

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

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

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

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

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

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

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

For further details on the requirements of each designated/elected Office, see Volume II of the PCT Applicant's Guide.

PATENT COOPERATION TREATY

PCT

INTERNATIONAL SEARCH REPORT

(PCT Article 18 and Rules 43 and 44)

Applicant's or agent's file reference	FOR FURTHER ACTION	See Notification of Transmittal of International Search Report (Form PCT/ISA/220) as well as, where applicable, item 5 below.
01107.00030		
International application No.	International filing date *(day/month/year)*	(Earliest) Priority Date *(day/month/year)*
PCT/US 00/20740	31/07/2000	02/08/1999
Applicant		
THE JOHNS HOPKINS UNIVERSITY et al.		

This International Search Report has been prepared by this International Searching Authority and is transmitted to the applicant according to Article 18. A copy is being transmitted to the International Bureau.

This International Search Report consists of a total of 5 sheets.

[X] It is also accompanied by a copy of each prior art document cited in this report.

1. **Basis of the report**

   a. With regard to the language, the international search was carried out on the basis of the international application in the language in which it was filed, unless otherwise indicated under this item.

   [ ] the international search was carried out on the basis of a translation of the international application furnished to this Authority (Rule 23.1(b)).

   b. With regard to any nucleotide and/or amino acid sequence disclosed in the international application, the international search was carried out on the basis of the sequence listing :

   [ ] contained in the international application in written form.

   [ ] filed together with the international application in computer readable form.

   [ ] furnished subsequently to this Authority in written form.

   [ ] furnished subsequently to this Authority in computer readble form.

   [ ] the statement that the subsequently furnished written sequence listing does not go beyond the disclosure in the international application as filed has been furnished.

   [ ] the statement that the information recorded in computer readable form is identical to the written sequence listing has been furnished

2. [ ] Certain claims were found unsearchable (See Box I).

3. [ ] Unity of invention is lacking (see Box II).

4. With regard to the title,

   [X] the text is approved as submitted by the applicant.

   [ ] the text has been established by this Authority to read as follows:

5. With regard to the abstract,

   [X] the text is approved as submitted by the applicant.

   [ ] the text has been established, according to Rule 38.2(b), by this Authority as it appears in Box III. The applicant may, within one month from the date of mailing of this international search report, submit comments to this Authority.

6. The figure of the drawings to be published with the abstract is Figure No. _____

   [ ] as suggested by the applicant.

   [ ] because the applicant failed to suggest a figure.

   [ ] because this figure better characterizes the invention.

   [X] None of the figures.

Form PCT/ISA/210 (first sheet) (July 1998)

INTERNATIONAL SEARCH REPORT

International Application No
PCT/US 00/20740

A. CLASSIFICATION OF SUBJECT MATTER
IPC 7 C12Q1/68

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

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 7 C12Q

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

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)
EPO-Internal, WPI Data, PAJ, BIOSIS, CHEM ABS Data, EMBASE, MEDLINE, SCISEARCH

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	VET JACQUELINE A M ET AL: "Multiplex detection of four pathogenic retroviruses using molecular beacons." PROCEEDINGS OF THE NATIONAL ACADEMY OF SCIENCES OF THE UNITED STATES, vol. 96, no. 11, 25 May 1999 (1999-05-25), pages 6394-6399, XP002145609 May 25, 1999 ISSN: 0027-8424 the whole document --- -/--	1-13, 15-19, 21-24, 30,32, 38-45, 47-51, 53-56, 62,64

[X] Further documents are listed in the continuation of box C.

[X] Patent family members are listed in annex.

* Special categories of cited documents :

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

"E" earlier document but published on or after the international filing date

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

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

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

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

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

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

"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
10 December 2001	21/12/2001

Name and mailing address of the ISA	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+31-70) 340-2040, Tx 31 651 epo nl, Fax: (+31-70) 340-3016	Gabriels, J

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Form PCT/ISA/210 (second sheet) (July 1992)

INTERNATIONAL SEARCH REPORT

International Application No
PCT/US 00/20740

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication,where appropriate, of the relevant passages	Relevant to claim No.
X	PIATEK AMY S ET AL: "Molecular beacon sequence analysis for detecting drug resistance in Mycobacterium tuberculosis." NATURE BIOTECHNOLOGY, vol. 16, no. 4, April 1998 (1998-04), pages 359-363, XP000891876 ISSN: 1087-0156 the whole document	1-13, 15-19, 21-24, 26,27, 30,32, 38-45, 47-51, 53-56, 58,59, 62,64
X	TYAGI SANJAY ET AL: "Multicolor molecular beacons for allele discrimination." NATURE BIOTECHNOLOGY, vol. 16, no. 1, January 1998 (1998-01), pages 49-53, XP002143901 ISSN: 1087-0156 the whole document	1-13, 15-19, 21-24, 26,27, 30,32, 38-45, 47-51, 53-56, 58,59, 62,64
X	TYAGI SANJAY ET AL: "Molecular beacons: Probes that fluoresce upon hybridization." NATURE BIOTECHNOLOGY, vol. 14, no. 3, 1996, pages 303-308, XP002914999 ISSN: 1087-0156 the whole document	1-19, 21-23, 30, 38-51, 53-55,62
X	WO 99 13113 A (KRAMER FRED R ;ALLAND DAVID (US); PIATEK AMY (US); TYAGI SANJAY (U) 18 March 1999 (1999-03-18) page 7, line 32 -page 9, line 3; claims 1-14; example 2	1-19, 21-23, 30, 38-51, 53-55,62
X	HALFORD WILLIAM P ET AL: "The inherent quantitative capacity of the reverse transcription-polymerase chain reaction." ANALYTICAL BIOCHEMISTRY, vol. 266, no. 2, 15 January 1999 (1999-01-15), pages 181-191, XP000791527 ISSN: 0003-2697 the whole document	1-12, 38-44
X	EP 0 643 140 A (CANON KK) 15 March 1995 (1995-03-15) page 1-3	1-12, 38-44

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Form PCT/ISA/210 (continuation of second sheet) (July 1992)

INTERNATIONAL SEARCH REPORT

International Application No
PCT/US 00/20740

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication,where appropriate, of the relevant passages	Relevant to claim No.
X	WO 95 13399 A (NEW YORK HEALTH RES INST) 18 May 1995 (1995-05-18) claims 1,2	33-37
A	US 5 804 383 A (GRUENERT DIETER C ET AL) 8 September 1998 (1998-09-08) claim 1	25,28, 57,60
A	US 5 858 663 A (NISSON PAUL E ET AL) 12 January 1999 (1999-01-12) claim 1	25,28, 57,60
A	DATABASE BIOSIS 'Online! BIOSCIENCES INFORMATION SERVICE, PHILADELPHIA, PA, US; April 1999 (1999-04) EVERETT KARIN D E ET AL: "Identification of nine species of the Chlamydiaceae using PCR-RFLP." Database accession no. PREV199900271658 XP002185145 abstract & INTERNATIONAL JOURNAL OF SYSTEMATIC BACTERIOLOGY, vol. 49, no. 2, April 1999 (1999-04), pages 803-813, ISSN: 0020-7713	20,52
P,X	VOGELSTEIN BERT ET AL: "Digital PCR." PROCEEDINGS OF THE NATIONAL ACADEMY OF SCIENCES OF THE UNITED STATES, vol. 96, no. 16, 3 August 1999 (1999-08-03), pages 9236-9241, XP002185144 Aug. 3, 1999 ISSN: 0027-8424 the whole document	1-64

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Form PCT/ISA/210 (continuation of second sheet) (July 1992)

INTERNATIONAL SEARCH REPORT

Information on patent family members

International Application No
PCT/US 00/20740

Patent document cited in search report		Publication date	Patent family member(s)		Publication date
WO 9913113	A	18-03-1999	AU	9484698 A	29-03-1999
			EP	1012344 A1	28-06-2000
			JP	2001515734 T	25-09-2001
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			AU	1292195 A	29-05-1995
			CA	2176348 A1	18-05-1995
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			AU	5087193 A	15-03-1994
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Form PCT/ISA/210 (patent family annex) (July 1992)

PATENT APPLICATION FEE DETERMINATION RECORD
Effective December 29, 1999

Application or Docket Number

CLAIMS AS FILED - PART I

FOR	(Column 1) NUMBER FILED		(Column 2) NUMBER EXTRA
BASIC FEE			
TOTAL CLAIMS	44	minus 20=	* 44
INDEPENDENT CLAIMS	5	minus 3 =	* 2
MULTIPLE DEPENDENT CLAIM PRESENT			

* If the difference in column 1 is less than zero, enter "0" in column 2

SMALL ENTITY TYPE ☐		OR	OTHER THAN SMALL ENTITY	
RATE	FEE		RATE	FEE
	345.00	OR		690.00
X$ 9=		OR	X$18=	792
X39=		OR	X78=	154
+130=		OR	+260=	
TOTAL		OR	TOTAL	1,136

CLAIMS AS AMENDED - PART II

AMENDMENT A

	(Column 1) CLAIMS REMAINING AFTER AMENDMENT		(Column 2) HIGHEST NUMBER PREVIOUSLY PAID FOR	(Column 3) PRESENT EXTRA
Total	*	Minus	**	=
Independent	*	Minus	***	=
FIRST PRESENTATION OF MULTIPLE DEPENDENT CLAIM				

SMALL ENTITY		OR	OTHER THAN SMALL ENTITY	
RATE	ADDITIONAL FEE		RATE	ADDITIONAL FEE
X$ 9=		OR	X$18=	
X39=		OR	X78=	
+130=		OR	+260=	
TOTAL ADDIT. FEE		OR	TOTAL ADDIT. FEE	

AMENDMENT B

	(Column 1) CLAIMS REMAINING AFTER AMENDMENT		(Column 2) HIGHEST NUMBER PREVIOUSLY PAID FOR	(Column 3) PRESENT EXTRA
Total	*	Minus	**	=
Independent	*	Minus	***	=
FIRST PRESENTATION OF MULTIPLE DEPENDENT CLAIM				

RATE	ADDITIONAL FEE		RATE	ADDITIONAL FEE
X$ 9=		OR	X$18=	
X39=		OR	X78=	
+130=		OR	+260=	
TOTAL ADDIT. FEE		OR	TOTAL ADDIT. FEE	

AMENDMENT C

	(Column 1) CLAIMS REMAINING AFTER AMENDMENT		(Column 2) HIGHEST NUMBER PREVIOUSLY PAID FOR	(Column 3) PRESENT EXTRA
Total	*	Minus	**	=
Independent	*	Minus	***	=
FIRST PRESENTATION OF MULTIPLE DEPENDENT CLAIM				

RATE	ADDITIONAL FEE		RATE	ADDITIONAL FEE
X$ 9=		OR	X$18=	
X39=		OR	X78=	
+130=		OR	+260=	
TOTAL ADDIT. FEE		OR	TOTAL ADDIT. FEE	

* If the entry in column 1 is less than the entry in column 2, write "0" in column 3.
** If the "Highest Number Previously Paid For" IN THIS SPACE is less than 20, enter "20."
*** If the "Highest Number Previously Paid For" IN THIS SPACE is less than 3, enter "3."
The "Highest Number Previously Paid For" (Total or Independent) is the highest number found in the appropriate box in column 1.

EXHIBIT 5

US005928870A

United States Patent [19]

Lapidus et al.

[11] **Patent Number: 5,928,870**

[45] **Date of Patent: *Jul. 27, 1999**

[54] **METHODS FOR THE DETECTION OF LOSS OF HETEROZYGOSITY**

[75] Inventors: **Stanley N. Lapidus**, Bedford, N.H.; **Anthony P. Shuber**, Milford, Mass.

[73] Assignee: **Exact Laboratories, Inc.**, Maynard, Mass.

[*] Notice: This patent is subject to a terminal disclaimer.

[21] Appl. No.: **08/876,857**

[22] Filed: **Jun. 16, 1997**

[51] **Int. Cl.**[6] **C12Q 1/68**

[52] **U.S. Cl.** **435/6**; 536/24.3

[58] **Field of Search** 435/6; 536/24.3

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0 390 323 A3	10/1990	European Pat. Off. .
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0 407 789 B1	1/1991	European Pat. Off. .
0 608 004 A2	7/1994	European Pat. Off. .
0 259 031 B1	11/1994	European Pat. Off. .
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WO 94/00603	1/1994	WIPO .
WO 94/09161	4/1994	WIPO .
WO 94/10575	5/1994	WIPO .
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WO 95/07361	3/1995	WIPO .
WO 95/09928	4/1995	WIPO .
WO 95/09929	4/1995	WIPO .
WO 95/12606	5/1995	WIPO .
WO 95/13397	5/1995	WIPO .
WO 95/15400	6/1995	WIPO .
WP 95/16792	6/1995	WIPO .
WO 95/18818	7/1995	WIPO .
WO 95/19448	7/1995	WIPO .
WO 95/25813	9/1995	WIPO .
WO 95/31728	11/1995	WIPO .
WO 96/01907	1/1996	WIPO .
WO 96/06951	3/1996	WIPO .
WO 96/08514	3/1996	WIPO .
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Attorney, Agent, or Firm—Testa, Hurwitz & Thibeault,LLP

[57] **ABSTRACT**

Methods are provided for detecting loss of heterozygosity in a nucleic acid sample. These methods are particularly useful for identifying individuals with gene mutations indicative of early colorectal cancer.

25 Claims, 3 Drawing Sheets

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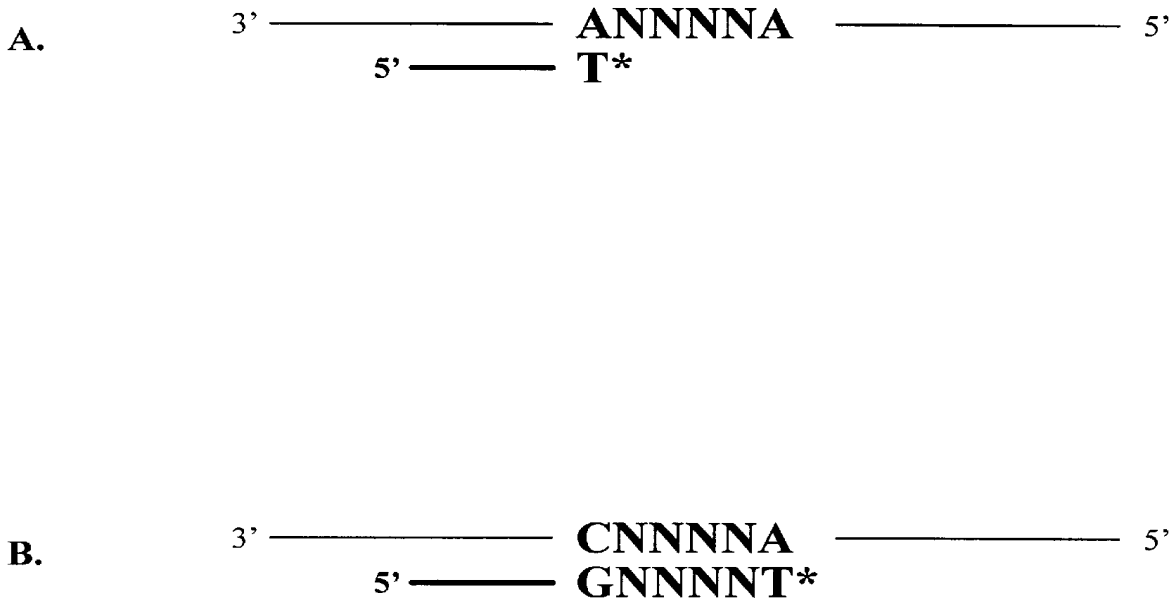


FIG. 1

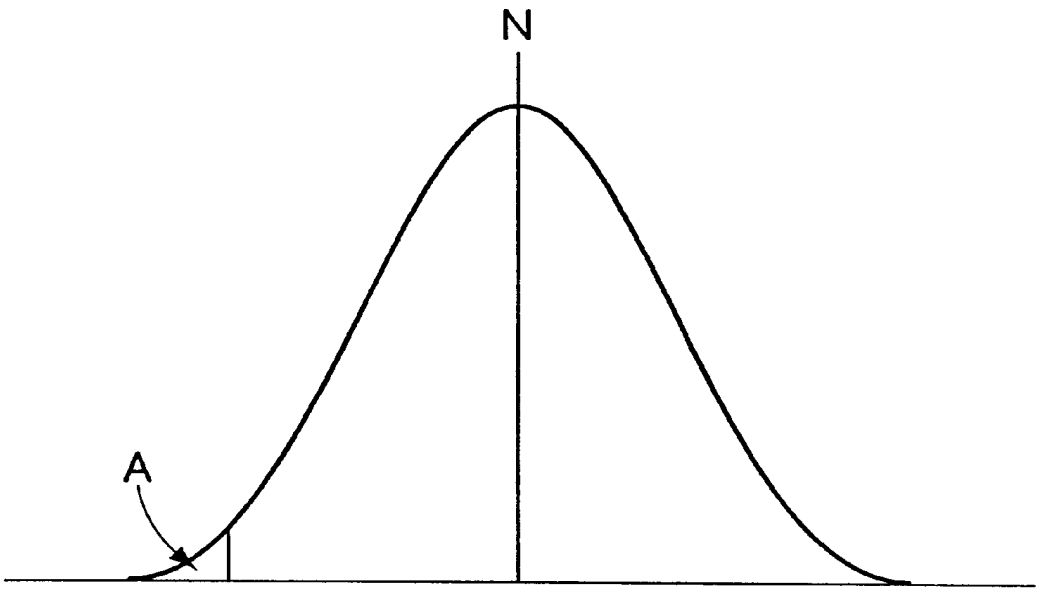


FIG. 2A

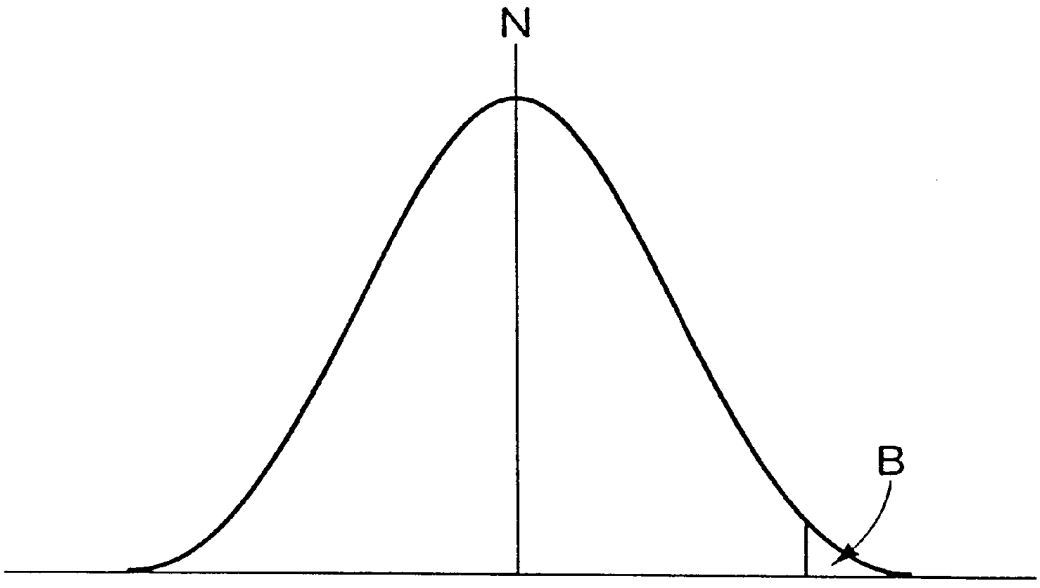


FIG. 2B

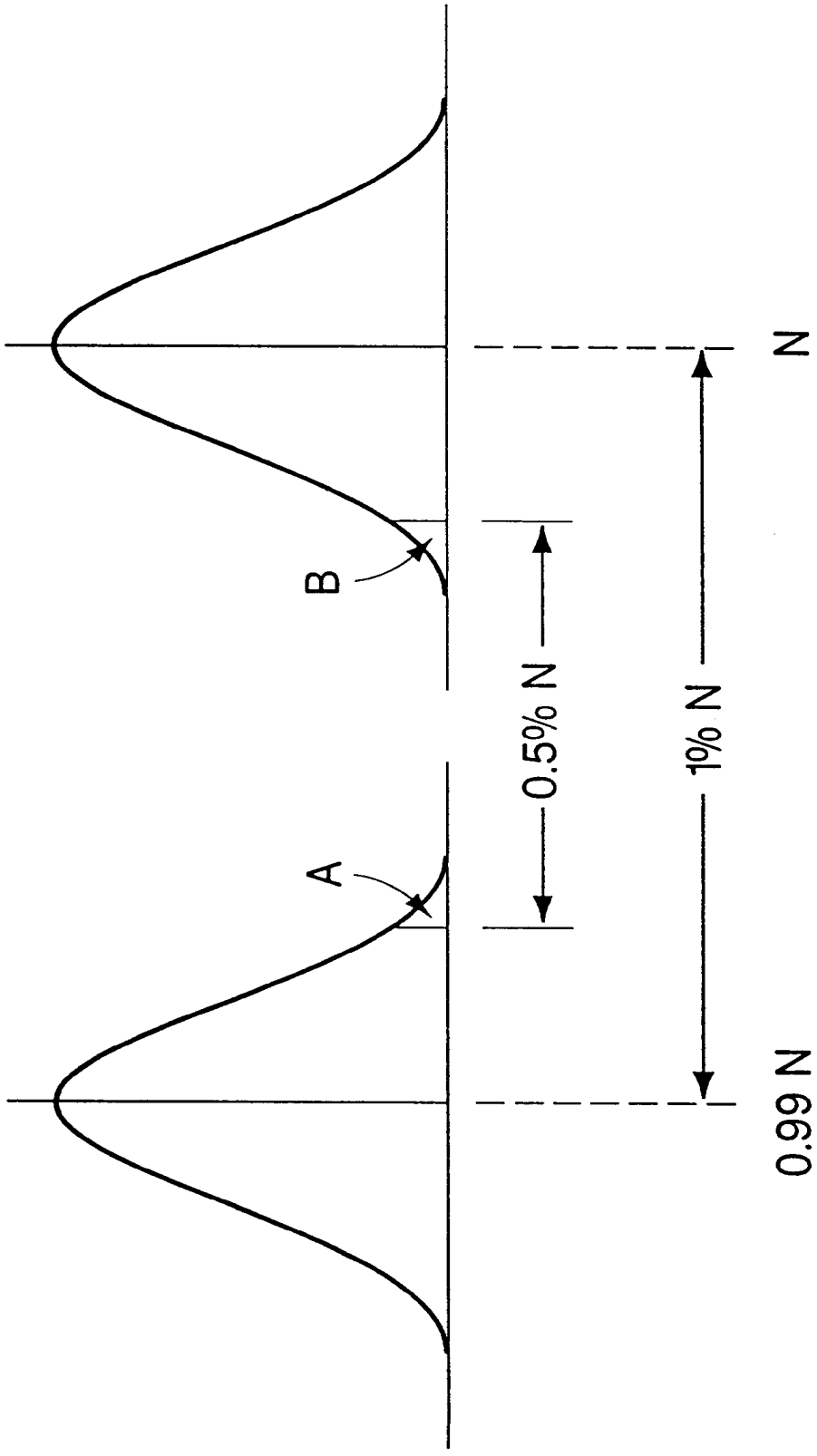


FIG. 3

METHODS FOR THE DETECTION OF LOSS OF HETEROZYGOSITY

FIELD OF THE INVENTION

This invention relates to methods useful for disease diagnosis by detecting loss of heterozygosity in cellular samples containing a small amount of mutated genetic material dispersed within a large amount of normal genetic material. Methods of the invention are especially useful in the detection of genetic mutations characteristic of cancer.

BACKGROUND OF THE INVENTION

Cancer is a disease characterized by genomic instability. The acquisition of genomic instability is thought to arise from a coincident disruption of genomic integrity and a loss of cell cycle control mechanisms. Generally, a disruption of genomic integrity is thought merely to increase the probability that a cell will engage in the multistep pathway leading to cancer. However, coupled with a loss of cell cycle control mechanisms, a disruption in genomic integrity may be sufficient to generate a population of genomically unstable neoplastic cells. A common genetic change characteristic of the early stages of transformation is loss of heterozygosity. Loss of heterozygosity at a number of tumor suppressor genes has been implicated in tumorigenesis. For example, loss of heterozygosity at the P53 tumor suppressor locus has been correlated with various types of cancer. Ridanpaa, et al., *Path. Res. Pract,* 191: 399–402 (1995). The loss of the apc and dcc tumor suppressor genes has also been associated with tumor development. *Blum, Europ. J. Cancer,* 31A: 1369–372 (1995).

Loss of heterozygosity is therefore a potentially useful marker for detecting the early stages of cancer. However, in the early stages of cancer only a small number of cells within a tissue have undergone transformation. Genetic changes characteristic of genomic instability theoretically can serve as markers for the early stages of, for example, colon cancer, and can be detected in DNA isolated from biopsied colonic epithelium and in some cases from transformed cells shed into fecal material. Sidransky, et al., *Science,* 256: 102–105 (1992).

Detection methods proposed in the art are time-consuming and expensive. Duffy, supra. Moreover, methods according to the art cannot be used to identify a loss of heterozygosity or microsatellite instability in small subpopulation of cells when the cells exist in a heterogeneous (i.e., clonally impure) sample. For example, in U.S. Pat. No. 5,527,676, it is stated that tissue samples in which a mutation is to be detected should be enriched for tumor cells in order to detect the loss of heterozygosity in a p53 gene.

Colorectal cancer is a common cause of death in Western society. Any tumor or precancerous polyp that develops along the length of the colon or the rectum sheds cells or DNA into the lumen of the colon. Shed cells or cellular DNA are usually incorporated onto and into stool as stool passes through the colon. In the early stages of cancer, cancerous or precancerous cells represent a very small fraction of the shed epithelial cells or DNA in stool. Current methods for detection of colorectal cancer do not focus on detecting cancerous or precancerous cells in stool. Rather, such methods typically focus on extracellular indicia of the presence of cancer, such as the presence of fecal occult blood or carcinoembryonic antigen circulating in serum.

It is thought that sporadic colorectal cancers result from mutations in oncogenes and tumor suppressor genes. Sporadic colorectal cancer is also typically associated with

massive loss of genetic material. Such mutations appear to occur at a point in the etiology of the disease that is much earlier than the point at which extracellular indicia or clinical signs of cancer are observed. If detected early, colon cancer may be effectively treated by surgical removal of the cancerous tissue. Surgical removal of early-stage colon cancer is usually successful because colon cancer begins in cells of the colonic epithelium and is isolated from the general circulation until the occurrence of invasion through the epithelial lining. Thus, detection of early mutations in colorectal cells would greatly increase survival rate.

Current non-invasive methods for detection of colon cancer involve the detection of fecal occult blood and carcinoembryonic antigen. These methods often either fail to detect colorectal cancer or they detect colorectal cancer only after it has progressed to a less treatable stage. Moreover, carcinoembryonic antigen is thought not to be an effective predictor of cancer but merely an indicator of recurrent cancer.

Invasive techniques, such as endoscopy, while effective, are expensive and painful and suffer from low patient compliance. Accordingly, current colon cancer screening methods are not practical for screening large segments of the population. See, e.g., *Blum, Europ. J. Cancer,* 31A: 1369–1372 (1995).

Therefore, there is a need in the art for simple and efficient non-invasive methods for reliable large-scale screening to identify individuals with early stage colon cancer. Such methods are provided herein.

SUMMARY OF THE INVENTION

The present invention provides methods for detecting a subpopulation of genomically transformed cells or cellular debris. Such methods detect the presence in a biological sample of a clonal subpopulation of cells which have a genome different from that of the wild type, and from bacterial, parasitic, or contaminating organisms that may also be present in the sample. Practice of the invention permits, for example, detection of a trace amount of DNA derived from cancer or precancer cells in a biological sample containing a majority of "normal" DNA. A preferred use of the methods is to reliably detect in a stool sample voided by a patient the presence of a trace amount of cells and/or cellular debris containing DNA shed into the colon at the site of an asymptomatic precancerous or cancerous lesion. The invention takes advantage of several important insights which permit, for example, reliable detection of a DNA deletion at a known genomic site characteristic of a known cancer cell type. Methods of the invention are useful for the detection and diagnosis of a genetic abnormality, such as a loss of heterozygosity or, more generally, a mutation, which can be correlated with a disease, such as cancer. For purposes of the present invention, unless the context requires otherwise, a "mutation" includes modifications, rearrangements, deletions, substitutions, and additions in a portion of genomic DNA or its corresponding mRNA.

In general, the invention comprises methods for counting (i.e. enumerating) the number of molecules of a target genomic sequence present in a sample. The invention further comprises methods for comparing the number of molecules with a reference number to determine whether any difference between the two numbers is statistically significant, a statistically significant difference being indicative of loss of heterozygosity involving a genomic region comprising the target sequence. A useful reference number is the number of molecules of a reference genomic sequence. The reference

genomic sequence is chosen such that the numbers of molecules of the target and reference genomic sequences are identical in normal cells which have not undergone loss of heterozygosity. When comparing the quantities of two genomic sequences in a sample, the enumerative methods are useful to identify a statistically-significant difference between the two quantities, and to correlate any difference, to a degree of defined statistical confidence, with the presence in the sample of a subpopulation of cells having an altered (e.g. Loss of heterozygosity) genomic sequence.

The invention may be divided into two general embodiments. (1) In a first general embodiment, an enumerative amount (number of copies) of a genetic region of interest in a sample (i.e. including a gene or genes, the mutation of which is known or suspected to be associated with cancer) is compared to an enumerative amount of a reference gene or gene fragment in the sample, the reference gene being a gene which is not normally associated with cancer and which normally has a low rate of mutation. A statistically-significant difference between the two enumerative amounts is indicative of genomic instability in a cellular subpopulation in the sample. (2) In a second general embodiment of the invention, an enumerative amount of a region on a maternal allele is compared to an enumerative amount of the corresponding region on a paternal allele. A statistically-significant difference between the two amounts is indicative of genomic instability.

In a preferred embodiment, enumerative detection of a nucleic acid mutation is accomplished by exposing a nucleic acid sample to first and second radionucleotides. The radionucleotides may be single nucleotides or oligonucleotide probes. The first radionucleotide is capable of hybridizing to a genetic region suspected to be mutated in cancer or precancer cells. The second radionucleotide is capable of hybridizing to a region known not to be mutated in cancer or precancer cells. After washing to remove unhybridized radionucleotides, the number of each of first and second radionucleotides is counted. A statistically-significant difference between the number of first and second radionucleotides is indicative of a mutation in a subpopulation of nucleic acids in the sample.

In preferred methods of the invention, first and second radionucleotides are isolated from other sample components by, for example, gel electrophoresis, chromatography, and mass spectrometry. Also in a preferred embodiment, either or both of the first and second radionucleotides is a chain terminator nucleotide, such as a dideoxy nucleotide. A preferred radionucleotide for use in methods of the invention is selected from the group consisting of ^{32}P, ^{33}P, ^{35}S, ^{3}H, ^{125}I, and ^{14}C. The number of first and second radionucleotides may be determined by counting. Methods of the invention are especially useful for the detection of massive nucleotide deletions, such as those that occur in loss of heterozygosity.

A massive loss of genetic material is detected as a reduction in the expected number in a sample of a nucleic acid fragment that is chosen to represent a genomic region suspected to be lost. For example, deletion of regions including all or part of human chromosome 18q have been associated with the development of cancer. According to the invention, a reduction in the number of cells in a sample having an intact 18q region is determined by comparing the number of a portion of the 18q region detected in the sample to the number of that region expected to occur in the sample. Similarly, a point mutation is detected by methods of the invention as a reduction in the sample of the number of wild-type nucleic acids encompassing the nucleotide suspected to be mutated. Accordingly, methods of the invention detect a mutation by detecting a reduction in the number of a nucleic acid expected to be in a sample. As described in detail below, methods of the invention are useful to detect a mutation in a heterogeneous cellular population without requiring the detection of multiple mutations.

An additional feature of the invention is that it has now been recognized that materials from cells lining the colon (e.g., a polyp or lesion) are shed onto forming stool only in a region comprising a longitudinal stripe along the length of the stool. Thus, unless the stool sample under investigation is a whole stool or comprises at least a cross-section of a stool, the sample will contain the relevant diagnostic information only by chance. The colon contains numerous bends and folds throughout its length. See, U.S. patent application Ser. No. 08/699,678 (Atty. Docket No. EXT-002), filed on Aug. 14, 1997. Epithelial cells lining the colon normally migrate from a basal position in colonic crypts, where stem cells divide by mitosis, to the top of the crypts and are then shed into the lumen. Colonic epithelial cells that line the intestinal lumen typically undergo regeneration every four to five days as a result of the rapid turnover rate through the epithelium. Accordingly, sloughed epithelial cells or their DNA are constantly being deposited in the forming stool as it passes through the lumen. As the stool proceeds toward the rectum and becomes progressively more solid (from an initial liquid state), epithelial cells are only sloughed onto the portion of the stool making contact with the portion of the lumen that formerly contained those cells in its epithelial lining. Epithelial cells of a polyp undergo the same rapid life cycle and shedding described above for normal colonic epithelial cells. Accordingly, cells shed from polyps are typically only absorbed onto the surface of the forming stool that makes contact with the polyp. However, if the stool is in a liquid state, mixing of shed polyp cells throughout the stool occurs automatically.

Accordingly, the present invention provides methods for detecting genomic changes in a subpopulation of cells in a sample of biological material. Methods of the invention are useful for the detection of changes in the nucleotide sequence of an allele in a small subpopulation of cells present in a large, heterogeneous sample of diagnostically-irrelevant biological material.

Also, in a preferred embodiment, transformed cells sought to be detected using methods according to the invention are malignant cells. Transformed cells detected according to methods of the invention may be induced transformants, transformed, for example, by a virus, by radiation, or by chemical or other carcinogenic means.

Methods of the invention may be performed on any biological sample, including tissue and body fluid samples. Particularly preferred biological samples include pus, sputum, semen, blood, saliva, cerebrospinal fluid, and urine. In an important embodiment of the invention the sample is stool which is analyzed to detect colorectal cancer or precancer. Methods of the invention may be practiced by exposing a biological sample to one or more radionucleotides in order to separately detect the number X of a first polynucleotide and the number Y of a second polynucleotide. In a preferred embodiment the radionucleotides are incorporated into oligonucleotide probes which are exposed to the sample under conditions that promote specific hybridization of the radiolabeled oligonucleotide probes with the first or second polynucleotides. In a more preferred embodiment, unlabeled oligonucleotide probes are exposed to the sample. The probes are subsequently radiolabeled using a primer extension reaction in the presence of radio-

labeled nucleotides. The radiolabeled nucleotides are preferably chain terminating nucleotides, (e.g. dideoxynucleotides). The number of molecules of a polynucleotide in a sample is calculated from the measurement of the number of radioactive decay events that is specifically associated with the polynucleotide. The number of radioactive decay events is directly proportional to the number of molecules.

In a preferred embodiment the first and second radiolabeled oligonucleotides are separable from each other. For example, the first and second oligonucleotides are of different sizes and can be separated by gel electrophoresis, chromatography or mass spectrometry. In one embodiment the first and second oligonucleotides are of different lengths. In a preferred embodiment the size difference is imparted by a size marker which is specifically attached to one of the different size marks. Alternatively a different size marker is attached to each oligonucleotide. After separation, the number of radioactive decay events is measured for each oligonucleotide, and the number of molecules is calculated as described herein.

In a more preferred embodiment, the first and second oligonucleotides are of the same size but are labeled with different radioisotopes selected from, for example, ^{35}S, ^{32}P, ^{33}P, ^{3}H, ^{125}I and ^{14}C. The first and second oligonucleotides are then distinguished by different characteristic emission spectra. The number of radioactive decay events is measured for each oligonucleotide without separating the two oligonucleotides from each other.

Methods of the invention are especially useful for the detection of colorectal cancer or precancerous cells in humans. For purposes of the present invention, precancerous cells are cells that have a mutation that is associated with cancer, and which renders such cells susceptible to becoming cancerous. Such methods comprise determining whether cells or nucleotide debris in a stool sample include a deletion of a polynucleotide normally present in a wild-type genome of the human or other mammal. The sample may be exposed to a plurality of first and second oligonucleotide probes under hybridization conditions, thereby to hybridize (i) first probe to copies of a first polynucleotide segment characteristic of a wild-type genomic region known or suspected not to be deleted in cells of the sample and (ii) second probe to copies of a second polynucleotide segment characteristic of the wild-type genomic region suspected of being mutated in the sample. The number of duplexes formed with each of the first and second probes is then detected and counted. The presence of a statistically-significant difference in those two numbers is indicative of the presence in the sample of a mutation that may be characteristic of colorectal cancer. Endoscopy or other visual examination procedures are then indicated.

Methods according to the invention also may be used to detect a loss of heterozygosity at an allele by determination of the amounts of maternal and paternal alleles comprising a genetic locus that includes at least one single-base polymorphism. A statistically-significant difference in the numbers of each allele is indicative of a mutation in an allelic region encompassing the single-base polymorphism. In this method, a region of an allele comprising a single-base polymorphism is identified, using, for example, a database, such as GenBank, or by other means known in the art. Probes are designed to hybridize to corresponding regions on both paternal and maternal alleles immediately 3' to the single base polymorphism. After hybridization, a mixture of at least two of the four common dideoxy nucleotides are added to the sample, each labeled with a different detectable label. A DNA polymerase is also added. Using allelic DNA adjacent the polymorphic nucleotide as a template, hybridized probe is extended by the addition of a single dideoxynucleotide that is the binding partner for the polymorphic nucleotide. After washing to remove unincorporated dideoxynucleotides, the dideoxynucleotides which have been incorporated into the probe extension are detected by determining the number of bound extended probes bearing each of the two dideoxy nucleotides in, for example, a scintillation counter. The presence of an almost equal number of two different labels mean that there is normal heterozygosity at the polymorphic nucleotide. The presence of a statistically-significant difference between the detected numbers of the two labels means that a deletion of the region encompassing the polymorphic nucleotide has occurred in one of the alleles.

Methods of the invention may be used to determine whether a patient is a candidate for follow-up invasive diagnostic or other procedures, such as endoscopy. For example, methods of the invention may be used to detect a mutation in a tumor suppressor gene or an oncogene in a subpopulation of cells in a stool sample obtained from a patient. An endoscopy procedure may then be performed on patients diagnosed with a mutation. A positive endoscopy result is then followed by polypectomy, surgery, or other treatment to remove cancerous or precancerous tissue.

Accordingly, it is an object of the invention to provide methods for detecting loss of heterozygosity in a subpopulation of cells in a cellular sample. It is a further object of the invention to provide methods for detecting a genomic change in a subpopulation of cells, wherein the genomic change is indicative of cancer. It is another object of the invention to detect a loss of heterozygosity in a genomic region associated with cancer, such as a tumor suppressor region. It is yet another object of the invention to provide methods for detecting heterozygosity and the loss thereof at single-base polymorphic nucleic acids. Finally, it is an object of the invention to provide methods for the detection of cancer, and particularly colorectal cancer by detection of cells or cellular debris indicative of cancer in a heterogeneous sample, such as a stool sample.

Further aspects of the invention will become apparent upon consideration of the following detailed description and of the drawings.

DESCRIPTION OF THE DRAWINGS

FIG. **1** depicts differential primer extension as exemplified below.

FIGS. **2A** and **2B** are model Gaussian distributions showing regions of low statistical probability.

FIG. **3** is graph showing the probable values of N for a heterogeneous population of cells in which 1% of the cells are mutated.

DETAILED DESCRIPTION OF THE INVENTION

Methods according to the present invention are useful for the detection of loss of heterozygosity in a heterogeneous cellular sample in which the loss of heterozygosity occurs in only a small subpopulation of cells in the sample. Using traditional detection methods, such a subpopulation would be difficult, if not impossible, to detect especially if the deletion end points are unknown at the time of detection or a clonally-impure cellular population is used. See, e.g., U.S. Pat. No. 5,527,676 (reporting that a clonal population of

cells should be used in order to detect a deletion in a p53 gene). Traditional methods for detection of mutations involved in carcinogenesis rely upon the use of a clonally-pure population of cells and such methods are best at detecting mutations that occur at known "hot spots" in oncogenes, such as k-ras. See, Sidransky, supra.

Methods of the present invention are useful for detecting loss of heterozygosity in a small number of cells in an impure cellular population because such methods do not rely upon knowing the precise deletion end-points and such methods are not affected by the presence in the sample of heterogeneous DNA. For example, in loss of heterozygosity, deletions occur over large portions of the genome and entire chromosome arms may be missing. Methods of the invention comprise counting a number of molecules of a target nucleic acid suspected of being deleted and comparing it to a reference number. In a preferred embodiment the reference number is the number of molecules of a nucleic acid suspected of not being deleted in the same sample. All that one needs to know is at least a portion of the sequence of a target nucleic acid suspected of being deleted and at least a portion of the sequence of a reference nucleic acid suspected of not being deleted. Methods of the invention, while amenable to multiple mutation detection, do not require multiple mutation detection in order to detect indicia of cancer in a heterogeneous sample.

Accordingly, methods of the present invention are useful for the detection of loss of heterozygosity in a subpopulation of cells or debris therefrom in a sample. Loss of heterozygosity generally occurs as a deletion of at least one wild-type allelic sequence in a subpopulation of cells. In the case of a tumor suppressor gene, the deletion typically takes the form of a massive deletion characteristic of loss of heterozygosity. Often, as in the case of certain forms of cancer, disease-causing deletions initially occur in a single cell which then produces a small subpopulation of mutant cells. By the time clinical manifestations of the mutation are detected, the disease may have progressed to an incurable stage. Methods of the invention allow detection of a deletion when it exists as only a small percentage of the total cells or cellular debris in a sample.

Methods of the invention comprise a comparison of the number of molecules of two nucleic acids that are expected to be present in the sample in equal numbers in normal (non-mutated) cells. In a preferred embodiment, the comparison is between (1) an amount of a genomic polynucleotide segment that is known or suspected not to be mutated in cells of the sample (the "reference") and (2) an amount of a wild-type (non-mutated) genomic polynucleotide segment suspected of being mutated in a subpopulation of cells in the sample (the "target"). A statistically-significant difference between the amounts of the two genomic polynucleotide segments indicates that a mutation has occurred.

In a preferred embodiment, the reference and target nucleic acids are alleles of the same genetic locus. Alleles are useful in methods of the invention if there is a sequence difference which distinguishes one allele from the other. In a preferred embodiment, the genetic locus is on or near a tumor suppressor gene. Loss of heterozygosity can result in loss of either allele, therefore either allele can serve as the reference allele. The important information is the presence or absence of a statistically significant difference between the number of molecules of each allele in the sample. Also in a preferred embodiment, the reference and target nucleic acids are different genetic loci, for example different genes. In a preferred embodiment, the reference nucleic acid comprises both alleles of a reference genetic locus and the target nucleic acid comprises both alleles of a target genetic locus, for example a tumor suppressor gene. Specifically, in the case of a deletion in a tumor suppressor gene, the detected amount of the reference gene is significantly greater than the detected amount of the target gene. If a target sequence is amplified, as in the case of certain oncogene mutations, the detected amount of target is greater than the detected amount of the reference gene by a statistically-significant margin.

Methods according to the art generally require the use of numerous probes, usually in the form of PCR primers and/or hybridization probes, in order to detect a deletion or a point mutation. However, because methods of the present invention involve enumerative detection of nucleotide sequences and enumerative comparisons between sequences that are known to be stable and those that are suspected of being unstable, only a few probes must be used in order to accurately assess cancer risk. In fact, a single set (pair) of probes is all that is necessary to detect a single large deletion. The risk of cancer is indicated by the presence of a mutation in a genetic region known or suspected to be involved in oncogenesis. Patients who are identified as being at risk based upon tests conducted according to methods of the invention are then directed to other, typically invasive, procedures for confirmation and/or treatment of the disease.

Enumerative sampling of a nucleotide sequence that is uniformly distributed in a biological sample typically follows a Poisson distribution. For large populations, such as the typical number of genomic polynucleotide segments in a biological sample, the Poisson distribution is similar to a normal (Gaussian) curve with a mean, N, and a standard deviation that may be approximated as the square root of N.

Statistically-significance between numbers of target and reference genes obtained from a biological sample may be determined by any appropriate method. See, e.g., Steel, et al., Principles and Procedures of Statistics, A Biometrical Approach (McGraw-Hill, 1980), the disclosure of which is incorporated by reference herein. An exemplary method is to determine, based upon a desired level of specificity (tolerance of false positives) and sensitivity (tolerance of false negatives) and within a selected level of confidence, the difference between numbers of target and reference genes that must be obtained in order to reach a chosen level of statistical significance. A threshold issue in such a determination is the minimum number, N, of genes (for each of target and reference) that must be available in a population in order to allow a determination of statistical significance. The number N will depend upon the assumption of a minimum number of mutant alleles in a sample containing mutant alleles (assumed herein to be at least 1%) and the further assumption that normal samples contain no mutant alleles. It is also assumed that a threshold differences between the numbers of reference and target genes must be at least 0.5% for a diagnosis that there is a mutation present in a subpopulation of cells in the sample. Based upon the foregoing assumptions, it is possible to determine how large N must be so that a detected difference between numbers of mutant and reference alleles of less than 0.5% is truly a negative (i.e. no mutant subpopulation in the sample) result 99.9% of the time.

The calculation of N for specificity, then, is based upon the probability of one sample measurement being in the portion of the Gaussian distribution covering the lowest 3.16% of the population (the area marked "A" in FIG. **2**A) and the probability that the other sample measurement is in the portion of the Gaussian distribution covering the highest 3.16% of the population (the area marked "B" in FIG. **2**B). Since the two sample measurements are independent events,

the probability of both events occurring simultaneously in a single sample is approximately 0.001 or 0.1%. Thus, 93.68% of the Gaussian distribution (100%−2×3.16%) lies between the areas marked A and B in FIG. **3**. Statistical tables indicate that such area is equivalent to 3.72 standard deviations. Accordingly, 0.5% N is set equal to 3.72 sigma. Since sigma (the standard deviation) is equal to $\sqrt{N}$, the equation may be solved for N as 553,536. This means that if the lower of the two numbers representing reference and target is at least 553,536 and if the patient is truly normal, the difference between the numbers will be less than 0.5% about 99.9% of the time.

To determine the minimum N required for 99% sensitivity a similar analysis is performed. This time, one-tailed Gaussian distribution tables show that 1.28 standard deviations (sigma) from the mean cover 90% of the Gaussian distribution. Moreover, there is a 10% (the square root of 1%) probability of one of the numbers (reference or target) being in either the area marked "A" in FIG. **3** or in the area marked "B" in FIG. **3**. If the two population means are a total of 1% different and if there must be a 0.5% difference between the number of target and reference genes, then the distance from either mean to the threshold for statistical significance is equivalent to 0.25% N (See FIG. **3**) for 99% sensitivity. As shown in FIG. **3**, 0.25% N corresponds to about 40% of one side of the Gaussian distribution. Statistical tables reveal that 40% of the Gaussian distribution corresponds to 1.28 standard deviations from the mean. Therefore, 1.28 sigma is equal to 0.0025N, and N equals 262,144. Thus, for abnormal samples, the difference will exceed 0.5% at least 99% of the time if the lower of the two numbers is at least 262,144. Conversely, an erroneous negative diagnosis will be made only 1% of the time under these conditions.

In order to have both 99.9% specificity (avoidance of false positives) and 99% sensitivity (avoidance of false negatives), a sample with DNA derived from at least 553, 536 (or roughly greater than 550,000) cells should be counted. A difference of at least 0.5% between the numbers obtained is significant at a confidence level of 99.0% for sensitivity and a difference of less than 0.5% between the numbers is significant at a confidence level of 99.9% for specificity. As noted above, other standard statistical tests may be used in order to determine statistical significance and the foregoing represents one such test.

Based upon the foregoing explanation, the skilled artisan appreciates that methods of the invention are useful to detect mutations in a subpopulation of a polynucleotides in any biological sample. For example, methods disclosed herein may be used to detect allelic loss (the loss of heterozygosity) associated with diseases such as cancer. Additionally, methods of the invention may be used to detect a deletion or a base substitution mutation causative of a metabolic error, such as complete or partial loss of enzyme activity. For purposes of exemplification, the following provides details of the use of methods according to the present invention in colon cancer detection. Inventive methods are especially useful in the early detection of a mutation (and especially a large deletion typical of loss of heterozygosity) in a tumor suppressor gene. Accordingly, while exemplified in the following manner, the invention is not so limited and the skilled artisan will appreciate its wide range of applicability upon consideration thereof.

Methods according to the invention preferably comprise comparing a number of a target polynucleotide known or suspected to be mutated to a number of a reference polynucleotide known or suspected not to be mutated. In addition to the alternative embodiments using either alleles or genetic loci as reference and target nucleic acids, the invention comprises a comparison of a microsatellite repeat region in a normal allele with the corresponding microsatellite region in an allele known or suspected to be mutated. Exemplary detection means of the invention comprise determining whether a difference exists between the number of counts of each nucleic acid being measured. The presence of a statistically-significant difference is indicative that a mutation has occurred in one of the nucleic acids being measured.

I. Preparation of a Stool Sample

A sample prepared from stool voided by a patient should comprise at least a cross-section of the voided stool. As noted above, stool is not homogenous with respect to sloughed cells. As stool passes through the colon, it absorbs sloughed cells from regions of the colonic epithelium with which it makes contacts. Thus, sloughed cells from a polyp are absorbed on only one surface of the forming stool (except near the cecum where stool is still liquid and is homogenized by Intestinal Peristalsis). Taking a representative sample of stool (i.e., at least a cross-section) and homogenizing it ensures that sloughed cells from all epithelial surfaces of the colon will be present for analysis in the processed stool sample. Stool is voided into a receptacle that is preferably small enough to be transported to a testing facility. The receptacle may be fitted to a conventional toilet such that the receptacle accepts stool voided in a conventional manner. The receptacle may comprise a mesh or a screen of sufficient size and placement such that stool is retained while urine is allowed to pass through the mesh or screen and into the toilet. The receptacle may additionally comprise means for homogenizing voided stool. Moreover, the receptacle may comprise means for introducing homogenization buffer or one or more preservatives, such as alcohol or a high salt concentration solution, in order to neutralize bacteria present in the stool sample and to inhibit degradation of DNA.

The receptacle, whether adapted to fit a toilet or simply adapted for receiving the voided stool sample, preferably has sealing means sufficient to contain the voided stool sample and any solution added thereto and to prevent the emanation of odors. The receptacle may have a support frame which is placed directly over a toilet bowl. The support frame has attached thereto an articulating cover which may be placed in a raised position, for depositing of sample or a closed position (not shown) for sealing voided stool within the receptacle. The support frame additionally has a central opening traversing from a top surface through to a bottom surface of the support frame. The bottom surface directly communicates with a top surface of the toilet. Extending from the bottom surface of the support frame and encompassing the entire circumference of the central opening is a means for capturing voided stool. The means for capturing voided stool may be fixedly attached to the support frame or may be removably attached for removal subsequent to deposition of stool.

Once obtained, the stool sample is homogenized in an appropriate buffer, such as phosphate buffered saline or a chaotropic salt solution. Homogenization means and materials for homogenization are generally known in the art. See, e.g., U.S. Pat. No. 4,101,279. Thus, particular homogenization methods may be selected by the skilled artisan. Methods for further processing and analysis of a biological sample, such as a stool sample are presented below.

II. Methods for Detection of Colon Cancer or Precancer

For exemplification, methods of the invention are used to detect a deletion or other mutation in or near the p53 tumor suppressor gene in cells obtained from a representative stool

sample. The p53 gene is a good choice because the loss of heterozygosity in p53 is often associated with colorectal cancer. An mRNA sequence corresponding to the DNA coding region for p53 is reported as GenBank Accession No. M92424. The skilled artisan understands that methods described herein may be used to detect mutations in any gene and that detection of a p53 deletion is exemplary of such methods. In the detection of loss of heterozygosity, it is not necessary to target any particular gene due to the massive deletions associated with this event. Accordingly, an LOH-type deletion involving, for example, p53 may be detected by probing a region outside, but near, p53 because that region is also likely to be deleted. At least a cross-section of a voided stool sample is obtained and prepared as described immediately above. DNA or RNA may optionally be isolated from the sample according to methods known in the art. See, Smith-Ravin, et al., *Gut,* 36: 81–86 (1995), incorporated by reference herein. Methods of the invention may also comprise the step of amplifying DNA or RNA sequences using the polymerase chain reaction. However, methods of the invention may be performed on unprocessed stool.

Nucleic acids may be sheared or cut into small fragments by, for example, restriction digestion. The size of nucleic acid fragments produced is not critical, subject to the limitations described below. A target nucleic acid that is suspected of being mutated (p53 in this example) and a reference nucleic acid are chosen. The target and reference nucleic acids may be alleles on or near the p53 gene. Alternatively, the target nucleic acid comprises both alleles on or near the p53 gene and the reference nucleic acid comprises both alleles on or near a genetic locus suspected not to be deleted. Single-stranded nucleic acid fragments may be prepared using well-known methods. See, e.g., Sambrook, et al., *Molecular Cloning, A Laboratory Manual* (1989) incorporated by reference herein.

Either portions of a coding strand or its complement may be detected in methods according to the invention. In a preferred embodiment, both first and second strands of an allele are present in a sample during hybridization to an oligonucleotide probe. The sample is exposed to an excess of probe that is complementary to a portion of the first strand, under conditions to promote specific hybridization of the probe to the portion of the first strand. In a most preferred embodiment, the probe is in sufficient excess to bind all the portion of the first strand, and to prevent reannealing of the first strand to the second strand of the allele. Also in a preferred embodiment, the second strand of an allele is removed from a sample prior to hybridization to an oligonucleotide probe that is complementary to a portion of the first strand of the allele. For exemplification, detection of the coding strand of p53 and reference allele are described. Complement to both p53 and reference allele are removed by hybridization to anti-complement oligonucleotide probes (isolation probes) and subsequent removal of duplex formed thereby. Methods for removal of complement strands from a mixture of single-stranded oligonucleotides are known in the art and include techniques such as affinity chromatography. Upon converting double-stranded DNA to single-stranded DNA, sample is passed through an affinity column comprising bound isolation probe that is complementary to the sequence to be isolated away from the sample. Conventional column chromatography is appropriate for isolation of complement. An affinity column packed with sepharose or any other appropriate materials with attached complementary nucleotides may be used to isolate complement DNA in the column, while allowing DNA to be analyzed to pass through the column. See Sambrook, Supra. As an alternative, isolation beads may be used to exclude complement as discussed in detail below.

After removal of complement, the target and reference nucleic acids are exposed to radio-labeled nucleotides under conditions which promote specific association of the radio-labeled nucleotides with the target and reference nucleic acids in a sample. In order to count the number of molecules of the target and reference nucleic acids, the radionucleotides associated with the target nucleic acid must be distinguished from the radionucleotides associated with the reference nucleic acid. In addition, the radionucleotides that are specifically associated with either target or reference nucleic acid must be distinguished from radionucleotides that are not associated with either nucleic acid. The number of molecules of target nucleic acid is counted by measuring a number X of radioactive decay events (e.g. by measuring the total number of counts during a defined interval or by measuring the time it takes to obtain a predetermined number of counts) specifically associated with the target nucleic acid. The number X is used to calculate the number X1 of radionucleotides which are specifically associated with the target nucleic acid. The number X1is used to calculate the number X2 of target nucleic acid molecules, knowing the ratio of radionucleotide molecules to target nucleic acid molecules in the assay.

According to methods of the invention, it is important to count the number of molecules in order to provide a statistical analysis of the likelihood of loss of heterozygosity. Comparison of the numbers of radioactive decays without knowing the numbers of molecules associated with the radioactive decays does not provide statistical data on the significance of any observed difference.

In a preferred embodiment, a radionucleotide is incorporated into a specific oligonucleotide prior to exposure to the sample. In a most preferred embodiment, a radiolabeled oligonucleotide is used which comprises a single radionucleotide molecule per oligonucleotide molecule. A radiolabeled oligonucleotide is designed to hybridize specifically to a target nucleic acid. In one embodiment the target nucleic acid is a specific allele of a polymorphic genetic locus, and the oligonucleotide is designed to be complementary to the allele at the site of polymorphism. One skilled in the art can perform hybridizations under conditions which promote specific hybridization of the oligonucleotide to the allele, without cross hybridizing to other alleles. Similarly, radiolabeled oligonucleotides are designed to specifically hybridize with the reference nucleic acid.

Also in a preferred embodiment, a radionucleotide is specifically incorporated into an oligonucleotide by primer extension, after exposing the oligonucleotide to the sample under conditions to promote specific hybridization of the oligonucleotide with the target nucleic acid. In a preferred embodiment the oligonucleotide is unlabeled, and the radionucleotide is a radiolabeled chain terminating nucleotide (e.g. a dideoxynucleotide). In a most preferred embodiment, the radionucleotide is the chain terminating nucleotide complementary to the nucleotide immediately 5' to the nucleotide that base pairs to the 3' nucleotide of the oligonucleotide when it is specifically hybridized to the target nucleic acid. In the embodiment where the target nucleic acid is an allele of a polymorphic genetic locus, the oligonucleotide is preferably designed such that the 3' nucleotide of the oligonucleotide base pairs with the nucleotide immediately 3' to the polymorphic residue. In a preferred embodiment, a radiolabeled terminating nucleotide that is complementary to the residue at the polymorphic site is

incorporated on the 3' end of the specifically hybridized oligonucleotide by a primer extension reaction. Similarly, in a preferred embodiment, a radionucleotide is specifically associated with a reference nucleic acid by primer extension. Other methods for specifically associating a radioactive isotope with a target or reference nucleic acid (for example a radiolabeled sequence specific DNA binding protein) are also useful for the methods of the invention.

In a preferred embodiment, prior to counting the radioactive decay events, the radionucleotides specifically associated with target and reference nucleic acids are separated from the radionucleotides that are not specifically associated with either nucleic acid. Separation is performed as described herein, or using techniques known in the art. Other separation techniques are also useful for practice of the invention. Methods of the invention also comprise distinguishing the radio-label specifically associated with a target nucleic acid from the radio-label specifically associated with a reference nucleic acid. In a preferred embodiment the isotope associated with the target is different from the isotope associated with the receptor. Different isotopes useful to radio-label nucleotides include ^{35}S, ^{32}P, ^{33}P, ^{125}I, ^{3}H, and ^{14}C. In one embodiment, an oligonucleotide complementary to a target nucleic acid is labeled with a different isotope from an oligonucleotide complementary to a reference nucleic acid. In another embodiment, the chain terminating nucleotide associated with the target nucleic acid is different from the chain terminating nucleotide associated with the reference nucleic acid, and the two chain terminating nucleotides are labeled with different isotopes.

In a preferred embodiment, radionucleotides labeled with different isotopes are detected without separating the radionucleotide associated with the target nucleic acid from the radionucleotide associated with the reference nucleic acid. The different isotopes useful to the invention have different characteristic emission spectra. The presence of a first isotope does not prevent the measurement of radioactive decay events of a second isotope. In a more preferred embodiment, the labeled oligonucleotide associated with the target nucleic acid is the same size as the labeled oligonucleotide associated with the reference nucleic acid (the labeled oligonucleotides can be labeled prior to hybridization or by primer extension). The two differentially labeled oligonucleotides are electrophoresed on a gel, preferably a denaturing gel, and the gel is exposed to an imager that detects the radioactive decay events of both isotopes. In this embodiment the two isotopes are detected at the same position on the imager, because both oligonucleotides migrate to the same position on the gel. Detection at the same position on the imager reduces variation due to different detection efficiencies at different positions on the imager.

Also in a preferred embodiment, the radionucleotide associated with the target nucleic acid is separated from the radionucleotide associated with the reference nucleic acid prior to measuring radioactive decay events. In a preferred embodiment the separated radionucleotides are labeled with the same isotope.

Preferred separation methods comprise conferring different molecular weights to the radionucleotides specifically associated with the target and reference nucleic acids.

In a preferred embodiment, first probes comprise a "separation moiety." Such separation moiety is, for example, hapten, biotin, or digoxigenin. The separation moiety in first probes does not interfere with the first probe's ability to hybridize with template or be extended. In an alternative embodiment, the labeled ddNTPs comprise a separation moiety. In yet another alternative embodiment, both the first probes and the labeled ddNTPs comprise a separation moiety. Following the extension reaction, a high molecular weight molecule having affinity for the separation moiety (e.g., avidin, streptavidin, or anti-digoxigenin) is added to the reaction mixture under conditions which permit the high molecular weight molecule to bind to the separation moiety. The reaction components are then separated on the basis of molecular weight using techniques known in the art such as gel electrophoresis, chromatography, or mass spectroscopy. See, Ausubel et al., *Short Protocols in Molecular Biology,* 3rd ed. (John Wiley & Sons, Inc., 1995); *Wu Recombinant DNA Methodology II,* (Academic Press, 1995).

Also in a preferred embodiment the radionucleotide associated with a first allele of a polymorphic genetic locus is separated from the radionucleotide associated with a second allele of the polymorphic locus by differential primer extension, wherein the extension products of a given oligonucleotide primer are of a different length for each of the two alleles. In differential primer extension (exemplified in FIG. **1**) an oligonucleotide is hybridized such that the 3' nucleotide of the oligonucleotide base pairs with the nucleotide that is immediately 5' of the polymorphic site. The extension reaction is performed in the presence of a radiolabeled terminator nucleotide complementary to the nucleotide at the polymorphic site of the first allele. The reaction also comprises non-labeled nucleotides complementary to the other 3 nucleotides. Extension of a primer hybridized to the first allele results in a product having only the terminator nucleotide incorporated (exemplified in FIG. **1A**, T* is the labeled terminator nucleotide). Extension of a primer hybridized to the second allele results in a product that incorporates several non-labeled nucleotides immediately 5' to the terminator nucleotide (exemplified in FIG. **1B**). The number of non-labeled nucleotides that are incorporated is determined by the position, on the template nucleic acid, of the closest 5' nucleotide complementary to the terminator nucleotide. In an alternative embodiment, differential primer extension comprises a labeled oligonucleotide and a non-labeled terminator nucleotide.

Labeled probes are exposed to sample under hybridization conditions. Such conditions are well-known in the art. See, e.g., Wallace, et al., *Nucleic Acids Res.,* 6: 3543–3557 (1979), incorporated by reference herein. First and Second oligonucleotide probes that are distinctly labeled (i.e. with different radioactive isotopes, fluorescent means, or with beads of different size) are applied to a single aliquot of sample. After exposure of the probes to sample under hybridization conditions, sample is washed to remove any unhybridized probe. Thereafter, hybridized probes are detected separately for p53 hybrids and reference allele hybrids. Standards may be used to establish background and to equilibrate results. Also, if differential fluorescent labels are used, the number of probes may be determined by counting differential fluorescent events in a sample that has been diluted sufficiently to enable detection of single fluorescent events in the sample. Duplicate samples may be analyzed in order to confirm the accuracy of results obtained.

If there is a difference between the amount of p53 detected and the amount of the reference allele detected greater than a 0.5% difference with at least 550,000 events (earlier shown to be the threshold of significance), it may be assumed that a mutation has occurred in the region involving p53 and the patient is at risk for developing or has developed colon cancer. Statistical significance may be determined by any known method. A preferred method is outlined above.

The determination of a p53 mutation allows a clinician to recommend further treatment, such as endoscopy procedures, in order to further diagnose and, if necessary, treat the patient's condition. The following examples illustrate methods of the invention that allow direct quantification of hybridization events.

What is claimed is:

1. A method for detecting the presence of a mutant nucleic acid in a sample population, comprising the steps of:

a) introducing a first radionucleotide to a sample population suspected to contain a subpopulation of a nucleic acid mutant, wherein said first radionucleotide hybridizes to a first wild-type nucleic acid target, a subpopulation of which suspected to be mutated in the sample;

b) introducing a second radionucleotide to the sample, wherein said second radionucleotide hybridizes to a second wild-type nucleic acid target in the sample

c) washing said sample to remove unhybridized first and second radionucleotides;

d) determining a number X of radioactive decay events associated with said first radionucleotide;

e) determining a number Y of radioactive decay events associated with said second radionucleotide;

f) determining whether a difference exists between number X and number Y, the presence of a statistically-significant difference being indicative of the presence of a mutation in said sample.

2. The method of claim **1** wherein said first radionucleotide is capable of hybridizing to a nucleic acid in the sample that is suspected to be mutated in cancer or precancer; and said second radionucleotide is capable of hybridizing to a nucleic acid in the sample that is not mutated in cancer or precancer.

3. The method of claim **1** wherein said first radionucleotide is capable of hybridizing to a portion of the maternal allele at a genetic locus; and said second radionucleotide is capable of hybridizing to a portion of the paternal allele at said locus.

4. The method of claim **1** further comprising the step of isolating said first radionucleotide specifically bound to a first target nucleic acid, and said second radionucleotide specifically bound to a second target nucleic acid.

5. The method of claim **4** wherein said isolating step is selected from the group consisting of gel electrophoresis, chromatography, and mass spectrometry.

6. The method of claim **4** wherein said number X is correlated with a number X1 of molecules of said first nucleic acid, and said number Y is correlated with a number Y1 of molecules of said second nucleic acid.

7. The method of claim **1** wherein at least one of said first and second radionucleotides is a chain terminator nucleotide.

8. The method of claim **1** wherein at least one of said first and second radionucleotides is an oligonucleotide.

9. The method of claim **1** wherein said radionucleotides are labeled with an isotope selected from the group consisting of 32P, 33P, 35S, 125I and 14C.

10. The method of claim **1** wherein each of said first and second radionucleotides are labeled with a different isotope.

11. The method of claim **10** wherein said numbers X and Y are determined by coincidence counting.

12. A method for determining the number of molecules of a nucleic acid comprising the steps of:

a) exposing a sample to a plurality of first radionucleotides;

b) isolating radionucleotides specifically bound to first target nucleic acid molecules;

c) determining a number of radioactive decay events associated with the radionucleotides of step b);

d) calculating a number of molecules of said sequence as equivalent to said number of radioactive decay events.

13. A method for detecting the presence of a mutation in a nucleic acid, comprising the steps of:

a) exposing a sample to a plurality of a oligonucleotide;

b) performing a primer extension reaction in the presence of a plurality of a chain terminating nucleotide, to generate extension products of said oligonucleotide;

c) determining the size of the extension products, the presence of extension products of different sizes being indicative of the presence of a mutation.

14. The method of claim **13** wherein said oligonucleotide is capable of hybridizing to a member selected from the group consisting of a maternal allele and a paternal allele of the same genetic locus.

15. The method of claim **13** wherein said oligonucleotide is labeled.

16. The method of claim **13** wherein said terminating nucleotide is labeled.

17. The method of claims **15** or **16** wherein said label is a radioactive isotope.

18. The method of claim **13** wherein said extension reaction is performed in the presence of at least two differentially labeled chain terminating nucleotides.

19. A method for detecting loss of heterozygosity in a nucleic acid, comprising the steps of:

a) contacting a sample with a radionucleotide;

b) isolating a nucleic acid specifically bound to said radionucleotide;

c) determining a number of radioactive decay events associated with said nucleic acid;

d) comparing said number to a reference number,

wherein a statistically significant difference between said number and said reference number is indicative of loss of heterozygosity.

20. The method of claim **1**, wherein said sample comprises cellular material from a population of patients.

21. The method of claim **20**, wherein said population of patients is healthy.

22. The method of claim **20**, wherein said population of patients has a disease suspected to be associated with said mutant nucleic acid.

23. The method of claim **20**, wherein said disease is cancer.

24. The method of claim **1**, wherein said mutant nucleic acid is an allelic variant.

25. The method of claim **24**, wherein said variant is a single nucleotide polymorphism.

* * * * *

UNITED STATES PATENT AND TRADEMARK OFFICE

CERTIFICATE OF CORRECTION

PATENT NO. : 5,928,870
APPLICATION NO. : 08/876857
DATED : July 27, 1999
INVENTOR(S) : Stanley Lapidus et al.

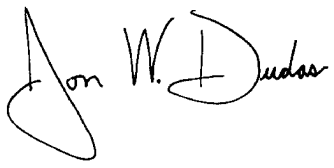
It is certified that error appears in the above-identified patent and that said Letters Patent is hereby corrected as shown below:

Column 1, between line 2, ending "HETEROZYGOSITY", and line 4, beginning "Field of", insert the following paragraph:

--This application is a continuation-in-part of U.S. Ser. No. 08/700,583, filed on August 14, 1996, now U.S. Pat. No. 5,670,325.--

Signed and Sealed this

Thirtieth Day of December, 2008



JON W. DUDAS
Director of the United States Patent and Trademark Office

EXHIBIT 7

US007915015B2

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

(10) **Patent No.: US 7,915,015 B2**
(45) **Date of Patent: *Mar. 29, 2011**

(54) **DIGITAL AMPLIFICATION**

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

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

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

This patent is subject to a terminal disclaimer.

(21) Appl. No.: **12/617,368**

(22) Filed: **Nov. 12, 2009**

(65) **Prior Publication Data**

US 2010/0209921 A1 Aug. 19, 2010

Related U.S. Application Data

(60) Division of application No. 11/709,742, filed on Feb. 23, 2007, now Pat. No. 7,824,889, which is a continuation of application No. 10/828,295, filed on Apr. 21, 2004, now abandoned, which is a division of application No. 09/981,356, filed on Oct. 12, 2001, now Pat. No. 6,753,147, which is a continuation of application No. 09/613,826, filed on Jul. 11, 2000, now Pat. No. 6,440,706.

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

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

(52) **U.S. Cl.** **435/91.2**; 536/24.3; 536/24.31; 536/24.33

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

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(57) **ABSTRACT**

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

18 Claims, 7 Drawing Sheets

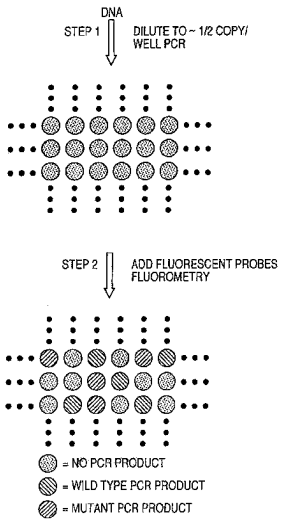

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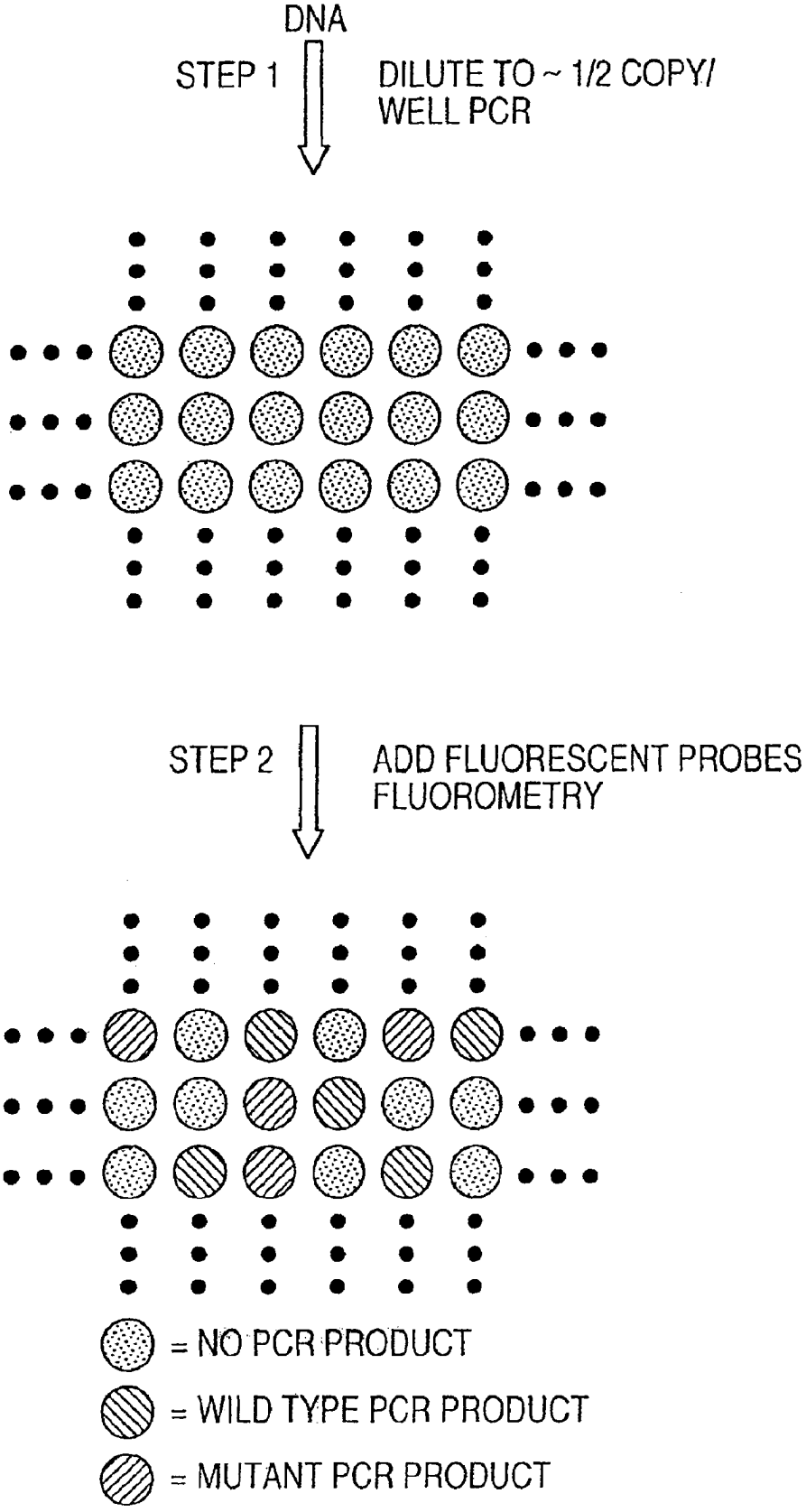
FIG. 1A


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

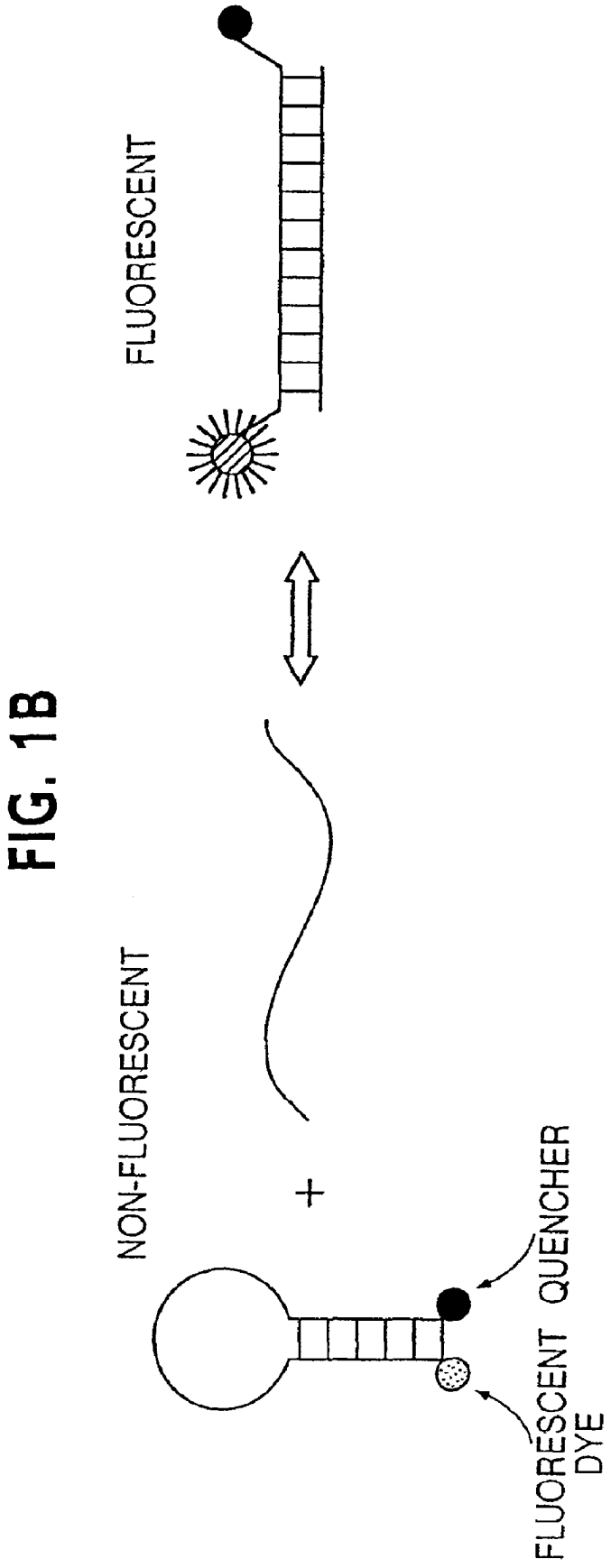


FIG. 1B

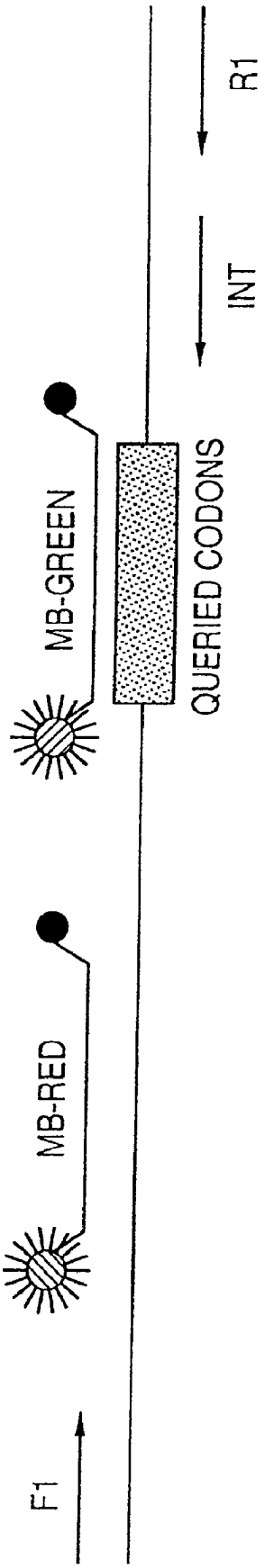


FIG. 1C

FIG. 2

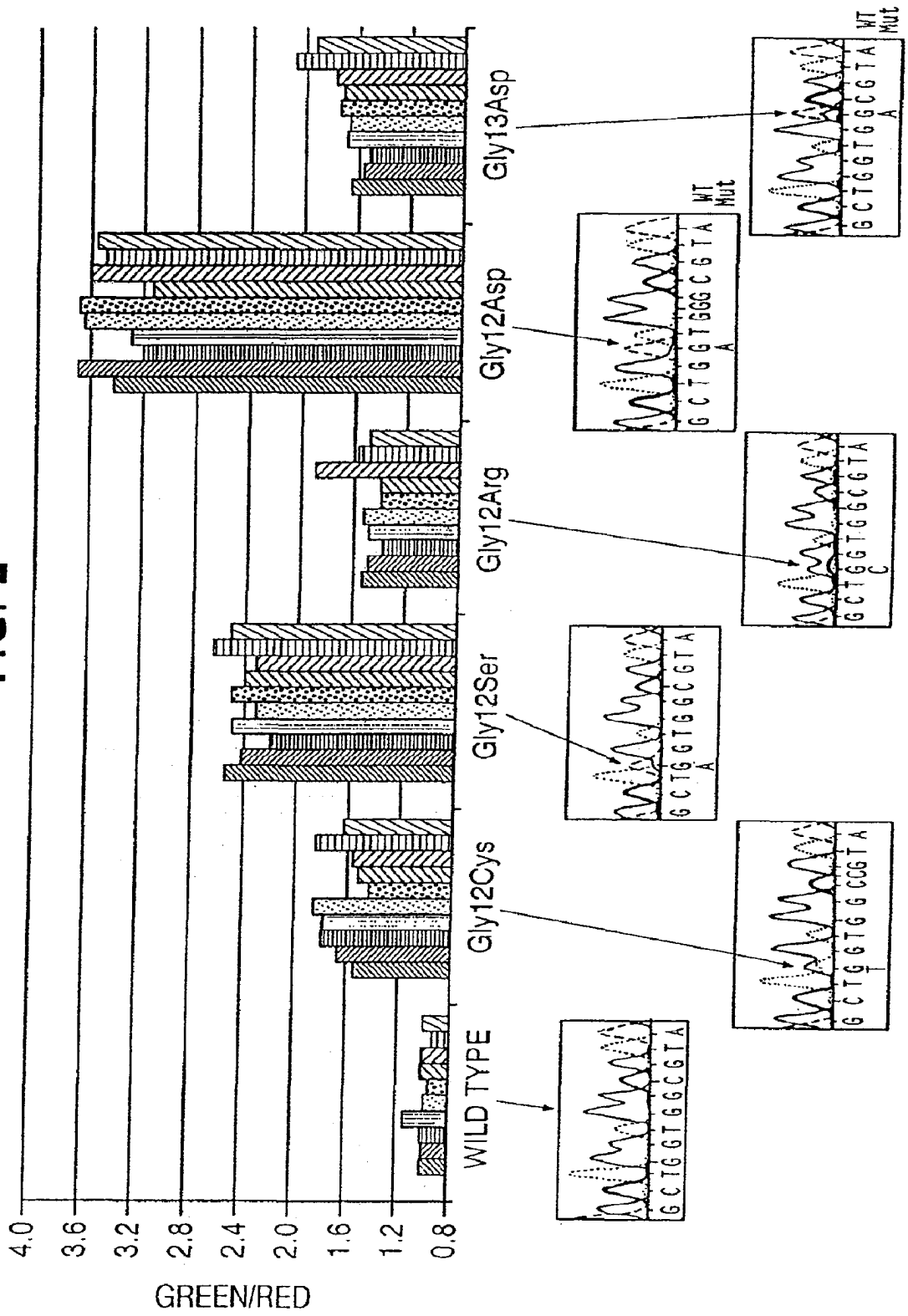
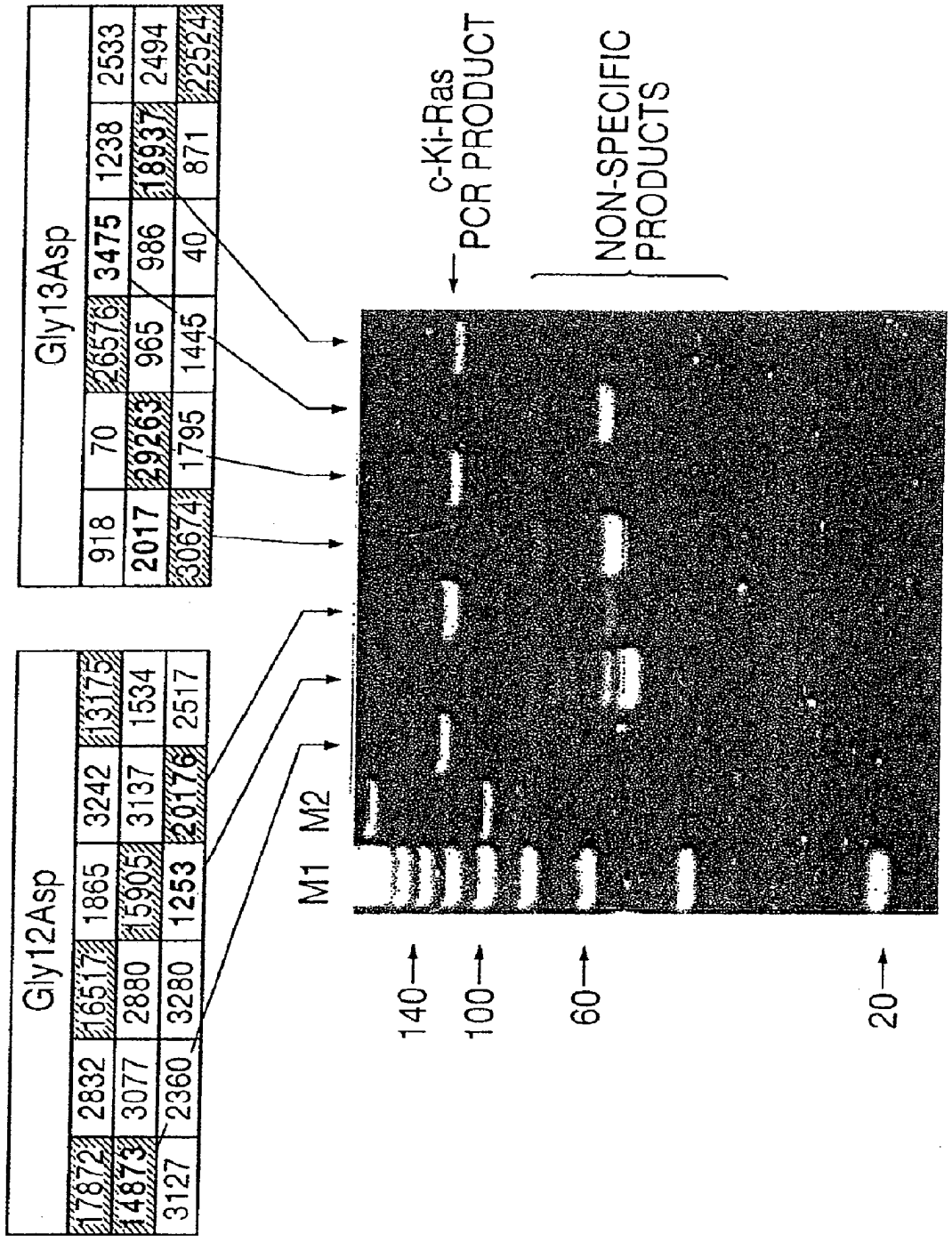

Gly13Asp
Gly12Asp
Gly12Arg
Gly12Ser
Gly12Cys
WILD TYPE
4.0
3.6
3.2
2.8
2.4
2.0
1.6
1.2
0.8
GREEN/RED
WT
Mut

FIG. 3


Gly12Asp
17872
2832
16517
1865
3242
13175
14873
3077
2880
5905
3137
1534
3127
2360
3280
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

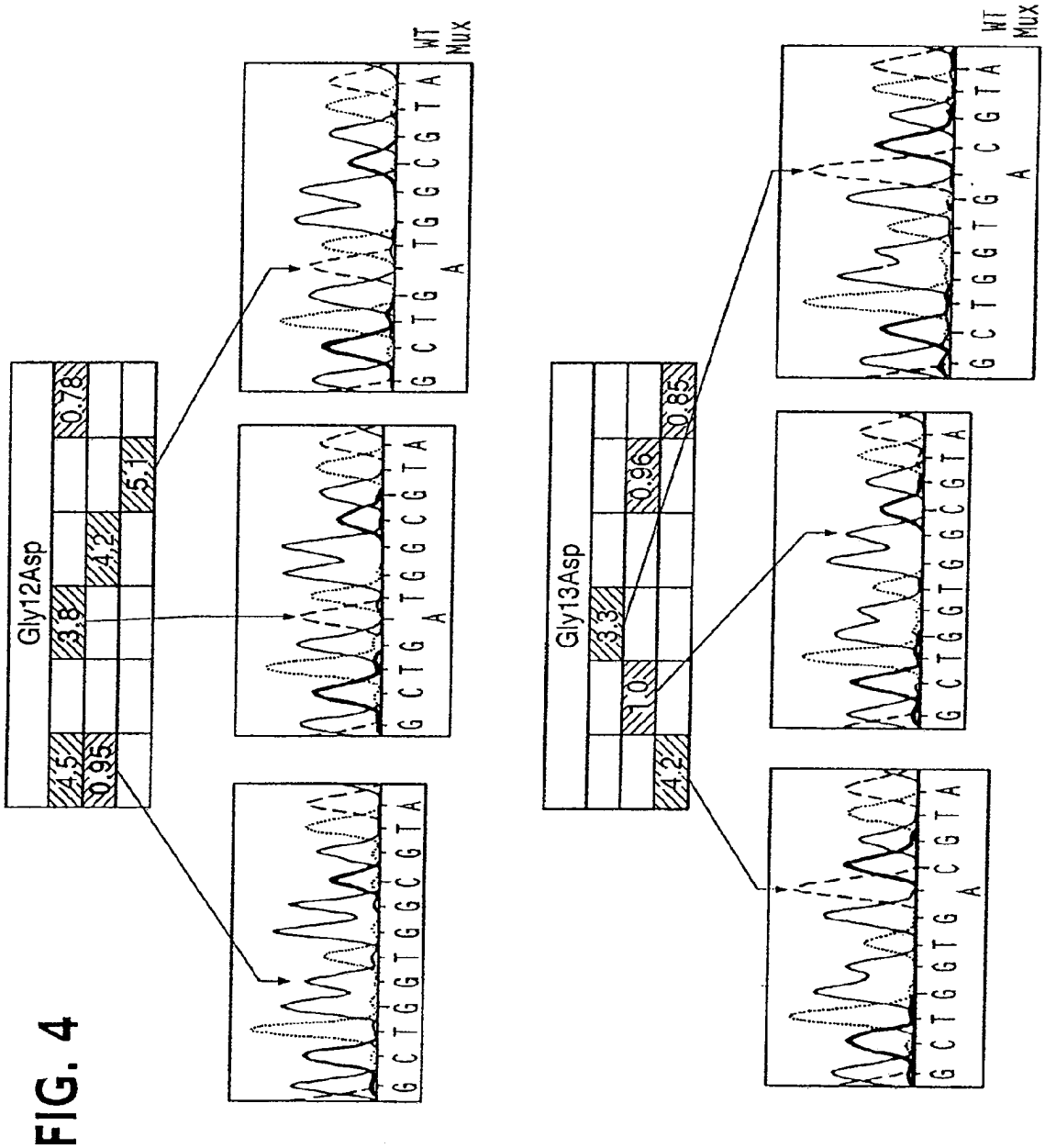
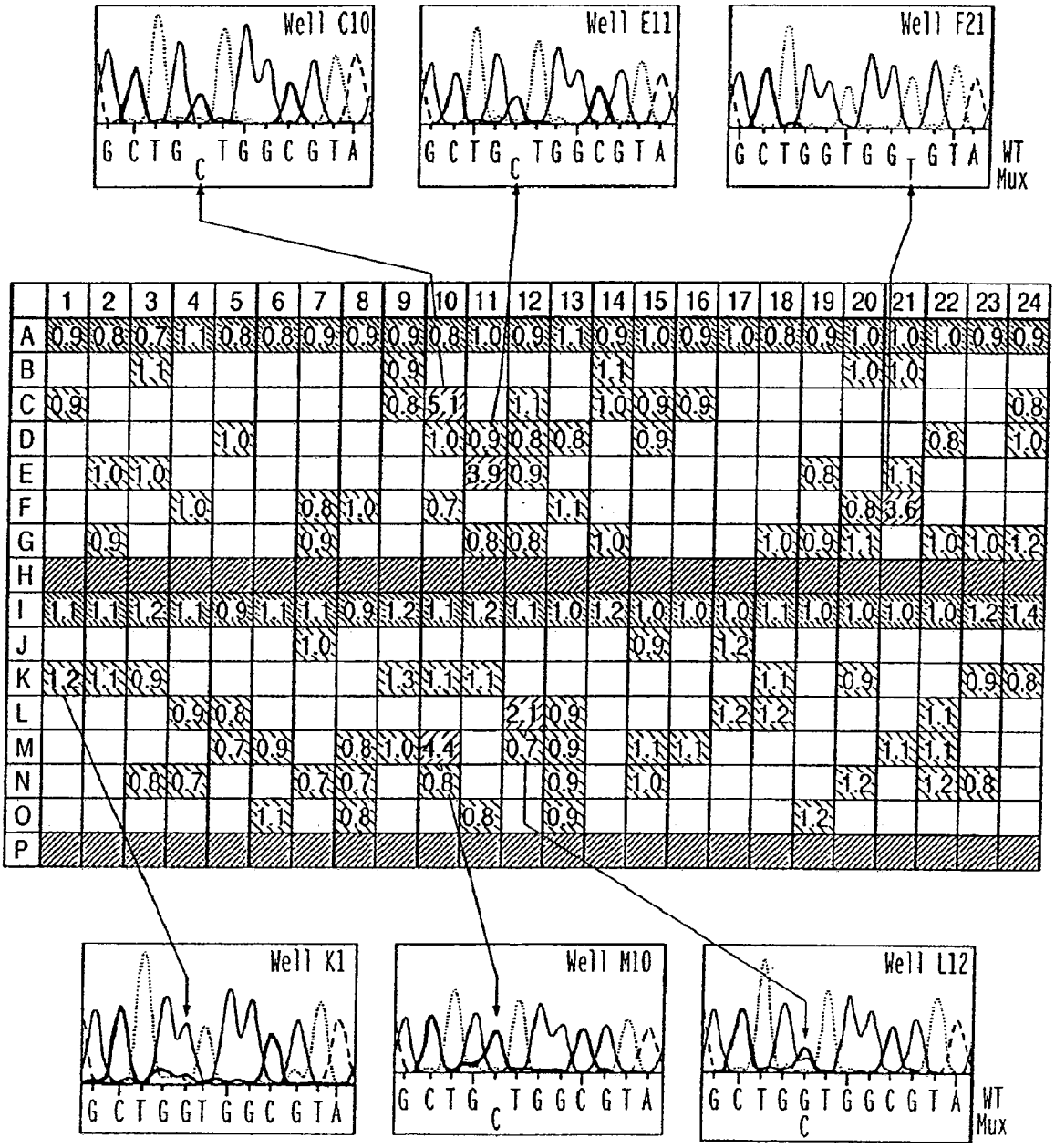

Gly12Asp
4.5
0.95
3.8
4.2
5.1
0.78
G C T G G C G T A
G C T G T G G C G T A
A
G C T G T G G C G T A
A
WT
MUX
Gly13Asp
4.2
1.0
3.3
0.96
0.85
G C T G G T G C G T A
G C T G G T G C G T A
A
G C T G G T G C G T A
A
WT
MUX

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).

SEQUENCE LISTING

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The invention claimed is:

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

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

analyzing the amplified molecules in the assay samples of the set to determine a first number of assay samples which contain a first allelic form of a marker and a second number of assay samples which contain a second allelic form of the marker, wherein between 0.1 and 0.9 of the assay samples yield an amplification product;

comparing the first number to the second number to ascertain an allelic imbalance in the biological sample; and

identifying an allelic imbalance in the biological sample.

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

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

4. The method of claim **1** wherein between 0.1 and 0.9 of the assay samples yield an amplification product as determined by amplification of the first allelic form of the marker.

5. The method of claim **1** wherein between 0.1 and 0.9 of the assay samples yield an amplification product as determined by amplification of the second allelic form of the marker.

6. The method of claim **1** wherein the amplified molecules in each of the assay samples within the first and second numbers of assay samples are homogeneous such that the first number of assay samples do not contain the second allelic form of the marker and the second number of assay samples do not contain the first allelic form of the marker.

7. The method of claim **1** wherein the sample is from blood.

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

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

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

analyzing the amplified molecules in the assay samples of the set to determine a first number of assay samples which contain a first allelic form of a marker and a second number of assay samples which contain a second allelic form of the marker;

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

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

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

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

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

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

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

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

16. The method of claim **8** wherein between 0.1 and 0.9 of the assay samples yield an amplification product as determined by amplification of the first allelic form of the marker.

17. The method of claim **8** wherein between 0.1 and 0.9 of the assay samples yield an amplification product as determined by amplification of the second allelic form of the marker.

18. The method of claim **8** wherein the amplified molecules in each of the assay samples within the first and second numbers of assay samples are homogeneous such that the first number of assay samples do not contain the second allelic form of the marker and the second number of assay samples do not contain the first allelic form of the marker.

* * * * *

EXHIBIT 11

PATENT

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

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

RESPONSE TO OFFICE ACITON

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

Sir:

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

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

IN THE CLAIMS

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

1. -48. (Cancelled)

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

Remarks

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

The rejection under § 112, second paragraph

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

Rejection under § 102(b)

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

Rejection under § 102(a)

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

The first rejection under § 103(a)

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

The second rejection under § 103(a)

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

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

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

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

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

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

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

Respectfully submitted,

Date: *March 11, 2013*

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

Banner & Witcoff, Ltd.
Customer No. 11332

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

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

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

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

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

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

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

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

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

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

WARNING: Information on this form may become public. Credit card information should not be included on this form. Provide credit card information and authorization on PTO-2038.

I am the

☐ applicant/inventor.

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

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

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

/Sarah A. Kagan/	11 March 2013
Signature	Date
Sarah A. Kagan	(202) 824-3000
Typed or printed name	Telephone Number

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

☑ Total of 1 forms are submitted.

Privacy Act Statement

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

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

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

Electronic Patent Application Fee Transmittal

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

Filed as Large Entity

Utility under 35 USC 111(a) Filing Fees

Description	Fee Code	Quantity	Amount	Sub-Total in USD($)
Basic Filing:				
Pages:				
Claims:				
Miscellaneous-Filing:				
Petition:				
Patent-Appeals-and-Interference:				
Post-Allowance-and-Post-Issuance:				
Extension-of-Time:				
Extension - 2 months with $0 paid	1252	1	570	570

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

Electronic Acknowledgement Receipt

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

Payment information:

Submitted with Payment	yes
Payment Type	Deposit Account
Payment was successfully received in RAM	$570
RAM confirmation Number	22590
Deposit Account	190733
Authorized User	
The Director of the USPTO is hereby authorized to charge indicated fees and credit any overpayment as follows:	
Charge any Additional Fees required under 37 C.F.R. Section 1.16 (National application filing, search, and examination fees)	
Charge any Additional Fees required under 37 C.F.R. Section 1.17 (Patent application and reexamination processing fees)	

Charge any Additional Fees required under 37 C.F.R. Section 1.19 (Document supply fees)

Charge any Additional Fees required under 37 C.F.R. Section 1.20 (Post Issuance fees)

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

File Listing:

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

Multipart Description/PDF files in .zip description

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

Warnings:

Information:

2	Extension of Time	Petition-for-EOT.PDF	289378 8919f47c7ad4711247bcaa1cf63e1efecb836292	no	2

Warnings:

Information:

3	Fee Worksheet (SB06)	fee-info.pdf	30272 92ce82a7a1eb8b0196183f2ecec64f34b3c693e2	no	2

Warnings:

Information:

Total Files Size (in bytes):	415612

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

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

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

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

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

PATENT APPLICATION FEE DETERMINATION RECORD

Substitute for Form PTO-875

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

APPLICATION AS FILED – PART I

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

APPLICATION AS AMENDED – PART II

AMENDMENT	03/11/2013	(Column 1) CLAIMS REMAINING AFTER AMENDMENT		(Column 2) HIGHEST NUMBER PREVIOUSLY PAID FOR	(Column 3) PRESENT EXTRA	SMALL ENTITY RATE ($)	ADDITIONAL FEE ($)	OR	OTHER THAN SMALL ENTITY RATE ($)	ADDITIONAL FEE ($)
	Total (37 CFR 1.16(i))	* 20	Minus	** 68	= 0	X $ =		OR	X $62=	0
	Independent (37 CFR 1.16(h))	* 1	Minus	***6	= 0	X $ =		OR	X $250=	0
	☐ Application Size Fee (37 CFR 1.16(s))									
	☐ FIRST PRESENTATION OF MULTIPLE DEPENDENT CLAIM (37 CFR 1.16(j))							OR		
						TOTAL ADD'L FEE		OR	TOTAL ADD'L FEE	0

AMENDMENT		(Column 1) CLAIMS REMAINING AFTER AMENDMENT		(Column 2) HIGHEST NUMBER PREVIOUSLY PAID FOR	(Column 3) PRESENT EXTRA	RATE ($)	ADDITIONAL FEE ($)	OR	RATE ($)	ADDITIONAL FEE ($)
	Total (37 CFR 1.16(i))	*	Minus	**	=	X $ =		OR	X $ =	
	Independent (37 CFR 1.16(h))	*	Minus	***	=	X $ =		OR	X $ =	
	☐ Application Size Fee (37 CFR 1.16(s))									
	☐ FIRST PRESENTATION OF MULTIPLE DEPENDENT CLAIM (37 CFR 1.16(j))							OR		
						TOTAL ADD'L FEE		OR	TOTAL ADD'L FEE	

* If the entry in column 1 is less than the entry in column 2, write "0" in column 3.

** If the "Highest Number Previously Paid For" IN THIS SPACE is less than 20, enter "20".

*** If the "Highest Number Previously Paid For" IN THIS SPACE is less than 3, enter "3".

The "Highest Number Previously Paid For" (Total or Independent) is the highest number found in the appropriate box in column 1.

Legal Instrument Examiner:
/SHARON HARRIS/

UNITED STATES PATENT AND TRADEMARK OFFICE

UNITED STATES DEPARTMENT OF COMMERCE
United States Patent and Trademark Office
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www.uspto.gov

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

11332 7590 10/10/2012

Banner & Witcoff, Ltd.
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1100 13th Street N.W.
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Washington, DC 20005-4051

EXAMINER	
WOOLWINE, SAMUEL C	

ART UNIT	PAPER NUMBER
1637	

MAIL DATE	DELIVERY MODE
10/10/2012	PAPER

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

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

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

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

Period for Reply

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

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

Status

1)☒ Responsive to communication(s) filed on *11 June 2012*.

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

3)☐ An election was made by the applicant in response to a restriction requirement set forth during the interview on _____; the restriction requirement and election have been incorporated into this action.

4)☐ Since this application is in condition for allowance except for formal matters, prosecution as to the merits is closed in accordance with the practice under *Ex parte Quayle*, 1935 C.D. 11, 453 O.G. 213.

Disposition of Claims

5)☒ Claim(s) *1-68* is/are pending in the application.

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

6)☐ Claim(s) _____ is/are allowed.

7)☒ Claim(s) *49-68* is/are rejected.

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

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

Application Papers

10)☐ The specification is objected to by the Examiner.

11)☐ The drawing(s) filed on _____ is/are: a)☐ accepted or b)☐ objected to by the Examiner.

Applicant may not request that any objection to the drawing(s) be held in abeyance. See 37 CFR 1.85(a).

Replacement drawing sheet(s) including the correction is required if the drawing(s) is objected to. See 37 CFR 1.121(d).

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

Priority under 35 U.S.C. § 119

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

a)☐ All b)☐ Some * c)☐ None of:

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

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

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

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

Attachment(s)

1) ☒ Notice of References Cited (PTO-892)
2) ☐ Notice of Draftsperson's Patent Drawing Review (PTO-948)
3) ☒ Information Disclosure Statement(s) (PTO/SB/08) Paper No(s)/Mail Date *03/24/2011*.
4) ☐ Interview Summary (PTO-413) Paper No(s)/Mail Date. _____ .
5) ☐ Notice of Informal Patent Application
6) ☐ Other: _____.

DETAILED ACTION

Election/Restrictions

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

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

Claim Rejections - 35 USC § 112

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

> The specification shall conclude with one or more claims particularly pointing out and distinctly claiming the subject matter which the applicant regards as his invention.

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

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

Claim Rejections - 35 USC § 102

The following is a quotation of the appropriate paragraphs of 35 U.S.C. 102 that form the basis for the rejections under this section made in this Office action:

> A person shall be entitled to a patent unless –
>
> (a) the invention was known or used by others in this country, or patented or described in a printed publication in this or a foreign country, before the invention thereof by the applicant for a patent.
>
> (b) the invention was patented or described in a printed publication in this or a foreign country or in public use or on sale in this country, more than one year prior to the date of application for patent in the United States.

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

With regard to claim 49, Li taught:

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

Claim Rejections - 35 USC § 103

The following is a quotation of 35 U.S.C. 103(a) which forms the basis for all obviousness rejections set forth in this Office action:

> (a) A patent may not be obtained though the invention is not identically disclosed or described as set forth in section 102 of this title, if the differences between the subject matter sought to be patented and the prior art are such that the subject matter as a whole would have been obvious at the time the invention was made to a person having ordinary skill in the art to which said subject matter pertains. Patentability shall not be negatived by the manner in which the invention was made.

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

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

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

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

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

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

The teachings of Irving have been discussed.

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

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

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

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

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

Conclusion

No claims are free of the prior art.

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

If attempts to reach the examiner by telephone are unsuccessful, the examiner's supervisor, Gary Benzion can be reached on (571) 272-0782. The

fax phone number for the organization where this application or proceeding is assigned is 571-273-8300.

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

/Samuel Woolwine/
Primary Examiner

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

U.S. PATENT DOCUMENTS

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

*		Include as applicable: Author, Title Date, Publisher, Edition or Volume, Pertinent Pages)
	U	Irving et al. TT Virus Infection in Patients with Hepatitis C: Frequency, Persistence, and Sequence Heterogeneity. The Journal of Infectious Diseases 180:27-34, July 1999.
	V	Simmonds et al. Human immunodeficiency virus-infected individuals contain provirus in small numbers of peripheral mononuclear cells and at low copy numbers. Journal of Virology 64(2):864-872 (1990).
	W	
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*A copy of this reference is not being furnished with this Office action. (See MPEP § 707.05(a).)
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Filing Date	2011-03-16
First Named Inventor	Bert Vogelstein et al.
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	3	5518901	A	1996-05-21	Murtagh	
	4	5804383	A	1998-09-08	Gruenert et al.	
	5	5858663	A	1999-01-12	Nisson et al.	
	6	5670325	A	1997-09-23	Lapidus et al.	
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	9	5928870	A	1999-07-27	Lapidus et al.	
	10	6020137	A	2000-02-01	Lapidus et al.	
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	1	LOUGHLIN ET AL., "Association of the Interleukin-1 Gene Cluster on Chromosome 2q13 With Knee Osteoarthritis," Arthritis & Rheumatism, June 2002, 46(6):1519-1527	☐
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	3	A. PIATEK ET AL., "Molecular Beacon Sequence Analysis for Detecting Drug Resistance in Mycobacterium Tuberculosis," Nature Biotechnology, April 1998, Vol. 16, No. 4. pp. 359-363	☐
	4	S. TYAGI ET AL., "Multicolor Molecular Beacons for allele discrimination," Nature Biotechnology, January 1998, Vol. 16, No. 1, pp. 303-308	☐
	5	J. A.M. VET ET AL., "Multilex Detection of Four Pathogenic Retroviruses Using Molecular Beacons," Proceedings of the National Academy of Sciences of the United States", May 25, 1999, Vol. 96, No. 11, pp. 6394-6399	☐
	6	S. TYAGI ET AL., "Molecular Beacons: probes that Fluoresce Upon Hybridization," Nature Biotechnology, 1996, Vol. 14, No. 3, pp. 303-308	☐
	7	W. P. HALFORD ET AL., "The Inherent Quantitative Capacity of the Reverse Transcription-Polymerase Chain Reaction," Analytical Biochemistry, January 15, 1999, Vol. 266, No. 2, pp. 181-191	☐
	8	B. VOGELSTEIN ET AL., "Digital PCR," Proceedings of the National Academy of Sciences of the United States, August 3, 1999, Vol. 96, No. 16, pp. 9236-9241	☐

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

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	20	MARRAS ET AL., "Multiplex Detection of Single-Nucleotide Variations Using Molecular Beacons," Genetic Analysis: Biomolecular Engineering, Feb. 1999, Vo. 14, pp. 151-156	☐
	21	WHITCOMB ET AL., "Detection of PCR Products Using Self-Probing Amplicons and Fluorescence," Nature Biotechnology, August 1999, Vol. 17, pp. 804-807	☐
	22	M.J. BRISCO ET AL., "Detection and Quantitation of Neoplastic Cells in Acute Lymphoblastic Leukemia, by Use of the Polymerase Chain Reaction," British Journal of Haematology, 1991, Vol. 79, pp. 211-217	☐
	23	M. J. BRISCO ET AL., "Outcome Prediction in Childhood Acute Lymphoblastic Leukemia by Molecular Quantification of Residual Disease at the End of Induction," The Lancet, January 22, 1994, Vol. 343, pp. 196-200	☐
	24	Notice of Reasons for Rejection dispatched April 28, 2010 in Japanese Application No. 2001-513641 and English translation thereof.	☐
	25	Stephens, J. Clairborne, et al. "Theoretical underpinning of the Single-Molecular-Dilution (SMD) Method of Direct Haplotype Resolution," Am. J. Hum. Gen., Vol. 46, pp. 1149-1155 (1990).	☐
	26	NEWTON, PCR Essential Data, pages 51-52, 1995	☐
	27	Office Action dated June 11, 2010, in co-pending application 11/709,742	☐
	28	Office Action dated December 29, 2009 in co-pending application 11/709,742	☐
	29	Office Action dated September 18, 2009 in co-pending application 11/709,742	☐
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L1	2449	(sample with (split splitting divide divided dividing dilute diluting diluted dilution)) same ((fragment molecule template) near5 (single one "less than" "more than" "greater than" "fewer than" fewer less))	US-PGPUB; USPAT; USOCR; FPRS; EPO; JPO; DERWENT; IBM_TDB	OR	ON	2012/10/02 15:01
L2	361	l1 and (@ad<"19990802" @pd<"19990802")	US-PGPUB; USPAT; USOCR; FPRS; EPO; JPO; DERWENT; IBM_TDB	OR	ON	2012/10/02 15:01
L3	12232	rare near5 (sequence target mutation variant variation polymorphism)	US-PGPUB; USPAT; USOCR; FPRS; EPO; JPO; DERWENT; IBM_TDB	OR	ON	2012/10/02 15:02
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S2	4	("7915015" "7824889" "6753147" "6440706").PN.	USPAT	OR	OFF	2012/10/01 07:49
S3	1	("20080287318").PN.	US-PGPUB; USPAT	OR	OFF	2012/10/01 15:54
S4	1132	"limiting dilution" same pcr	US-PGPUB; USPAT; USOCR; FPRS; EPO; JPO; DERWENT; IBM_TDB	OR	OFF	2012/10/01 16:52
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			JPO; DERWENT; IBM_TDB			
S7	26	S5 and (sequencing sequenced) with ((pcr amplification) near2 product)	US-PGPUB; USPAT; USOCR; FPRS; EPO; JPO; DERWENT; IBM_TDB	OR	ON	2012/10/01 17:05
S8	4	(vogelstein kinzler).in. and (dilut$3 distribut$3).clm. and (sequencing (determin$5 near2 sequence)).clm. and (samples aliquots portions tubes wells).clm.	US-PGPUB; USPAT; USOCR; FPRS; EPO; JPO; DERWENT; IBM_TDB	OR	ON	2012/10/01 17:13
S9	289	((distributing diluting aliquotting dividing splitting) with (dna nucleic sample)) same (amplif$7 pcr) same (sequencing sequenced)	US-PGPUB; USPAT; USOCR; FPRS; EPO; JPO; DERWENT; IBM_TDB	OR	ON	2012/10/01 17:31
S10	16	S9 and (@ad<"19990802" @pd<"19990802")	US-PGPUB; USPAT; USOCR; FPRS; EPO; JPO; DERWENT; IBM_TDB	OR	OFF	2012/10/01 17:31
S11	1337	((distributing diluting aliquotting dividing splitting) with (dna nucleic sample)) same (amplif$7 pcr) and (sequencing sequenced)	US-PGPUB; USPAT; USOCR; FPRS; EPO; JPO; DERWENT; IBM_TDB	OR	ON	2012/10/01 17:32
S12	312	S11 and ((less fewer) near5 molecules)	US-PGPUB; USPAT; USOCR; FPRS; EPO; JPO; DERWENT; IBM_TDB	OR	ON	2012/10/01 17:33
S13	8	S12 and (@ad<"19990802" @pd<"19990802")	US-PGPUB; USPAT; USOCR; FPRS; EPO; JPO; DERWENT; IBM_TDB	OR	OFF	2012/10/01 17:33
S18	506	(rare adj1 (sequence target mutation)) and ((pcr "polymerase chain") same (dilution diluting diluted))	US-PGPUB; USPAT; USOCR; FPRS; EPO; JPO; DERWENT; IBM_TDB	OR	ON	2012/10/01 22:55
S19	56	S18 and (@ad<"19990802" @pd<"19990802")	US-PGPUB; USPAT;	OR	ON	2012/10/01 22:55

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

10/ 2/ 2012 3:45:50 PM
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EAST Search History

EAST Search History (Prior Art)

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

10/ 2/ 2012 5:51:13 PM
C:\ Users\ swoolwine\ Documents\ EAST\ Workspaces\ 13071105.wsp

PATENT

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

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

RESPONSE TO RESTRICTION REQUIREMENT

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

Sir:

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

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

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

IN THE CLAIMS

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

identifying an allelic imbalance based on the ratio ascertained.

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

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

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

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

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

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

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

identifying an allelic imbalance based on the ratio ascertained.

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

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

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

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

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

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

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

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

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

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

identifying an allelic imbalance based on the ratio ascertained.

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

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

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

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

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

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

identifying an allelic imbalance based on the ratio ascertained.

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

Respectfully submitted,

Date: June 11, 2012

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

Banner & Witcoff, Ltd.
Customer No. 11332

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

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

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

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

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

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

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

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

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

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

WARNING: Information on this form may become public. Credit card information should not be included on this form. Provide credit card information and authorization on PTO-2038.

I am the

☐ applicant/inventor.

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

☑ attorney or agent of record. Registration Number 32141

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

/Sarah A. Kagan/ — Signature

June 11, 2012 — Date

Sarah A. Kagan — Typed or printed name

(202) 824-3000 — Telephone Number

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

☑ Total of 1 forms are submitted.

Privacy Act Statement

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

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

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

Electronic Patent Application Fee Transmittal

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

Filed as Large Entity

Utility under 35 USC 111(a) Filing Fees

Description	Fee Code	Quantity	Amount	Sub-Total in USD($)
Basic Filing:				
Pages:				
Claims:				
Miscellaneous-Filing:				
Petition:				
Patent-Appeals-and-Interference:				
Post-Allowance-and-Post-Issuance:				
Extension-of-Time:				
Extension - 1 month with $0 paid	1251	1	150	150

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

Electronic Acknowledgement Receipt

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

Payment information:

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

File Listing:

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

1		response_to_RR.pdf	83294 abfb45b23bf7dbe6e6d49bd5fb4b0d6ae1758080	yes	10
	Multipart Description/PDF files in .zip description				
	Document Description		Start	End	
	Response to Election / Restriction Filed		1	1	
	Claims		2	10	
Warnings:					
Information:					
2	Extension of Time	EOT_filed_with_RR.pdf	286857 1f2225aaa12244d9c218066b5f8025f32bfb15fb	no	2
Warnings:					
Information:					
3	Fee Worksheet (SB06)	fee-info.pdf	30091 07c17696daf0ce93600409d2e67488efe9daf944	no	2
Warnings:					
Information:					
		Total Files Size (in bytes):	400242		

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

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

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

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

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

PATENT APPLICATION FEE DETERMINATION RECORD
Substitute for Form PTO-875

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

APPLICATION AS FILED – PART I

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

APPLICATION AS AMENDED – PART II

AMENDMENT 06/11/2012

	(Column 1) CLAIMS REMAINING AFTER AMENDMENT		(Column 2) HIGHEST NUMBER PREVIOUSLY PAID FOR	(Column 3) PRESENT EXTRA	SMALL ENTITY RATE ($)	SMALL ENTITY ADDITIONAL FEE ($)	OR	OTHER THAN SMALL ENTITY RATE ($)	OTHER THAN SMALL ENTITY ADDITIONAL FEE ($)
Total (37 CFR 1.16(i))	* 68	Minus	** 68	= 0	X $ =		OR	X $60=	0
Independent (37 CFR 1.16(h))	* 6	Minus	***6	= 0	X $ =		OR	X $250=	0
☐ Application Size Fee (37 CFR 1.16(s))									
☐ FIRST PRESENTATION OF MULTIPLE DEPENDENT CLAIM (37 CFR 1.16(j))							OR		
					TOTAL ADD'L FEE		OR	TOTAL ADD'L FEE	0

AMENDMENT

	(Column 1) CLAIMS REMAINING AFTER AMENDMENT		(Column 2) HIGHEST NUMBER PREVIOUSLY PAID FOR	(Column 3) PRESENT EXTRA	RATE ($)	ADDITIONAL FEE ($)	OR	RATE ($)	ADDITIONAL FEE ($)
Total (37 CFR 1.16(i))	*	Minus	**	=	X $ =		OR	X $ =	
Independent (37 CFR 1.16(h))	*	Minus	***	=	X $ =		OR	X $ =	
☐ Application Size Fee (37 CFR 1.16(s))									
☐ FIRST PRESENTATION OF MULTIPLE DEPENDENT CLAIM (37 CFR 1.16(j))							OR		
					TOTAL ADD'L FEE		OR	TOTAL ADD'L FEE	

* If the entry in column 1 is less than the entry in column 2, write "0" in column 3.
** If the "Highest Number Previously Paid For" IN THIS SPACE is less than 20, enter "20".
*** If the "Highest Number Previously Paid For" IN THIS SPACE is less than 3, enter "3".
The "Highest Number Previously Paid For" (Total or Independent) is the highest number found in the appropriate box in column 1.

Legal Instrument Examiner:
/KIM P. DOZIER/

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

11332 7590 04/10/2012

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EXAMINER	
WOOLWINE, SAMUEL C	

ART UNIT	PAPER NUMBER
1637	

MAIL DATE	DELIVERY MODE
04/10/2012	PAPER

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

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

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

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

Period for Reply

A SHORTENED STATUTORY PERIOD FOR REPLY IS SET TO EXPIRE 1 MONTH(S) OR THIRTY (30) DAYS, WHICHEVER IS LONGER, FROM THE MAILING DATE OF THIS COMMUNICATION.

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

Status

1)☐ Responsive to communication(s) filed on _____.

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

3)☐ An election was made by the applicant in response to a restriction requirement set forth during the interview on _____; the restriction requirement and election have been incorporated into this action.

4)☐ Since this application is in condition for allowance except for formal matters, prosecution as to the merits is closed in accordance with the practice under *Ex parte Quayle*, 1935 C.D. 11, 453 O.G. 213.

Disposition of Claims

5)☒ Claim(s) 1-68 is/are pending in the application.

5a) Of the above claim(s) _____ is/are withdrawn from consideration.

6)☐ Claim(s) _____ is/are allowed.

7)☐ Claim(s) _____ is/are rejected.

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

9)☒ Claim(s) 1-68 are subject to restriction and/or election requirement.

Application Papers

10)☐ The specification is objected to by the Examiner.

11)☐ The drawing(s) filed on _____ is/are: a)☐ accepted or b)☐ objected to by the Examiner.

Applicant may not request that any objection to the drawing(s) be held in abeyance. See 37 CFR 1.85(a).

Replacement drawing sheet(s) including the correction is required if the drawing(s) is objected to. See 37 CFR 1.121(d).

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

Priority under 35 U.S.C. § 119

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

a)☐ All b)☐ Some * c)☐ None of:

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

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

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

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

Attachment(s)

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

DETAILED ACTION

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

Election/Restrictions

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

The election of an invention may be made with or without traverse. To reserve a right to petition, the election must be made with traverse. If the reply does not distinctly and specifically point out supposed errors in the restriction requirement, the election shall be treated as an election without traverse. Traversal must be presented at the time of election in order to be considered timely. Failure to timely traverse the requirement will result in the loss of right to petition under 37 CFR 1.144. If claims are added after the election, applicant must indicate which of these claims are readable upon the elected invention.

Should applicant traverse on the ground that the inventions are not patentably distinct, applicant should submit evidence or identify such evidence now of record showing the inventions to be obvious variants or clearly admit on the record that this is the case. In either instance, if the examiner finds one of the inventions unpatentable over the prior art, the evidence or admission may be used in a rejection under 35 U.S.C. 103(a) of the other invention.

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

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

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

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

B2: analyzing employs gel electrophoresis (claim 13).

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

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

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

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

D1: mutation is translocated allele (claim 25).

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

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

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

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

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

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

Applicant is advised that the reply to this requirement to be complete must include (i) an election of a species or a grouping of patentably indistinct species to be examined even though the requirement may be traversed (37 CFR 1.143) **and (ii) identification of the claims encompassing the elected species or grouping of patentably indistinct species**, including any claims subsequently added. An argument that a claim is allowable or that all claims are generic is considered nonresponsive unless accompanied by an election.

The election may be made with or without traverse. To preserve a right to petition, the election must be made with traverse. If the reply does not distinctly and specifically point out supposed errors in the election of species requirement, the election shall be treated as an election without traverse. Traversal must be presented at the time of election in order to be considered timely. Failure to timely traverse the requirement will result in the loss of right to petition under 37 CFR 1.144. If claims are added after the election, applicant must indicate which of these claims are readable on the elected species or grouping of patentably indistinct species.

Should applicant traverse on the ground that the species, or groupings of patentably indistinct species from which election is required, are not patentably distinct,

applicant should submit evidence or identify such evidence now of record showing them to be obvious variants or clearly admit on the record that this is the case. In either instance, if the examiner finds one of the species unpatentable over the prior art, the evidence or admission may be used in a rejection under 35 U.S.C. 103(a) of the other species.

Upon the allowance of a generic claim, applicant will be entitled to consideration of claims to additional species which depend from or otherwise require all the limitations of an allowable generic claim as provided by 37 CFR 1.141.

Applicant is reminded that upon the cancellation of claims to a non-elected invention, the inventorship must be amended in compliance with 37 CFR 1.48(b) if one or more of the currently named inventors is no longer an inventor of at least one claim remaining in the application. Any amendment of inventorship must be accompanied by a request under 37 CFR 1.48(b) and by the fee required under 37 CFR 1.17(i).

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

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

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

/Samuel Woolwine/
Primary Examiner

UTILITY PATENT

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

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

For: DIGITAL AMPLIFICATION

PRELIMINARY AMENDMENT

U.S. Patent and Trademark Office
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Dear Sir:

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

IN THE CLAIMS

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

Remarks

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

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

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

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

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

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

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

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

Respectfully submitted,

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

Customer No. 11332

Dated: November 30, 2011

Electronic Patent Application Fee Transmittal

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

Filed as Large Entity

Utility under 35 USC 111(a) Filing Fees

Description	Fee Code	Quantity	Amount	Sub-Total in USD($)
Basic Filing:				
Pages:				
Claims:				
Claims in excess of 20	1202	20	60	1200
Independent claims in excess of 3	1201	1	250	250
Miscellaneous-Filing:				
Petition:				
Patent-Appeals-and-Interference:				
Post-Allowance-and-Post-Issuance:				

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

Electronic Acknowledgement Receipt

EFS ID:	11509270
Application Number:	13071105
International Application Number:	
Confirmation Number:	3361
Title of Invention:	Digital Amplification
First Named Inventor/Applicant Name:	Bert VOGELSTEIN
Customer Number:	11332
Filer:	Sarah Anne Kagan.
Filer Authorized By:	
Attorney Docket Number:	001107.00866
Receipt Date:	30-NOV-2011
Filing Date:	24-MAR-2011
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Application Type:	Utility under 35 USC 111(a)

Payment information:

Submitted with Payment	yes
Payment Type	Deposit Account
Payment was successfully received in RAM	$1450
RAM confirmation Number	3
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	Preliminary Amendment	001107prelim00866amd.pdf	112622 2d05049633ecde45742e71819abd860f17e bd9f5	no	17
Warnings:					
Information:					
2	Fee Worksheet (SB06)	fee-info.pdf	31434 c6b0ee1754b1d8e29ac3e2d1306b90b2ce2 fa060	no	2
Warnings:					
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		Total Files Size (in bytes):	144056		

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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
13/071,105	03/24/2011	

APPLICATION AS FILED – PART I

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

APPLICATION AS AMENDED – PART II

AMENDMENT	11/30/2011	(Column 1) CLAIMS REMAINING AFTER AMENDMENT		(Column 2) HIGHEST NUMBER PREVIOUSLY PAID FOR	(Column 3) PRESENT EXTRA	SMALL ENTITY RATE ($)	SMALL ENTITY ADDITIONAL FEE ($)	OR	OTHER THAN SMALL ENTITY RATE ($)	OTHER THAN SMALL ENTITY ADDITIONAL FEE ($)
	Total (37 CFR 1.16(i))	* 68	Minus	** 48	= 20	X $ =		OR	X $60=	1200
	Independent (37 CFR 1.16(h))	* 6	Minus	***5	= 1	X $ =		OR	X $250=	250
	☐ Application Size Fee (37 CFR 1.16(s))									
	☐ FIRST PRESENTATION OF MULTIPLE DEPENDENT CLAIM (37 CFR 1.16(j))							OR		
						TOTAL ADD'L FEE		OR	TOTAL ADD'L FEE	1450

AMENDMENT		(Column 1) CLAIMS REMAINING AFTER AMENDMENT		(Column 2) HIGHEST NUMBER PREVIOUSLY PAID FOR	(Column 3) PRESENT EXTRA	RATE ($)	ADDITIONAL FEE ($)	OR	RATE ($)	ADDITIONAL FEE ($)
	Total (37 CFR 1.16(i))	*	Minus	**	=	X $ =		OR	X $ =	
	Independent (37 CFR 1.16(h))	*	Minus	***	=	X $ =		OR	X $ =	
	☐ Application Size Fee (37 CFR 1.16(s))									
	☐ FIRST PRESENTATION OF MULTIPLE DEPENDENT CLAIM (37 CFR 1.16(j))							OR		
						TOTAL ADD'L FEE		OR	TOTAL ADD'L FEE	

* If the entry in column 1 is less than the entry in column 2, write "0" in column 3.

** If the "Highest Number Previously Paid For" IN THIS SPACE is less than 20, enter "20".

*** If the "Highest Number Previously Paid For" IN THIS SPACE is less than 3, enter "3".

The "Highest Number Previously Paid For" (Total or Independent) is the highest number found in the appropriate box in column 1.

Legal Instrument Examiner:
/ELMIRA HALL/

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

OC000000030585019

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:

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PART 1 - ATTORNEY/APPLICANT COPY
page 1 of 1

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

CONFIRMATION NO. 3361

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

PUBLICATION NOTICE

Title: Digital Amplification

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

NOTICE OF PUBLICATION OF APPLICATION

The above-identified application will be electronically published as a patent application publication pursuant to 37 CFR 1.211, et seq. The patent application publication number and publication date are set forth above.

The publication may be accessed through the USPTO's publically available Searchable Databases via the Internet at www.uspto.gov. The direct link to access the publication is currently http://www.uspto.gov/patft/.

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APPLICATION NUMBER	FILING or 371(c) DATE	GRP ART UNIT	FIL FEE REC'D	ATTY.DOCKET.NO	TOT CLAIMS	IND CLAIMS
13/071,105	03/24/2011	1634	2986	001107.00866	48	5

CONFIRMATION NO. 3361

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UPDATED FILING RECEIPT

Date Mailed: 07/15/2011

Receipt is acknowledged of this non-provisional patent application. The application will be taken up for examination in due course. Applicant will be notified as to the results of the examination. Any correspondence concerning the application must include the following identification information: the U.S. APPLICATION NUMBER, FILING DATE, NAME OF APPLICANT, and TITLE OF INVENTION. Fees transmitted by check or draft are subject to collection. Please verify the accuracy of the data presented on this receipt. **If an error is noted on this Filing Receipt, please submit a written request for a Filing Receipt Correction. Please provide a copy of this Filing Receipt with the changes noted thereon. If you received a "Notice to File Missing Parts" for this application, please submit any corrections to this Filing Receipt with your reply to the Notice. When the USPTO processes the reply to the Notice, the USPTO will generate another Filing Receipt incorporating the requested corrections**

Applicant(s)

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Assignment For Published Patent Application

THE JOHNS HOPKINS UNIVERSITY, Baltimore, MD

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Domestic Priority data as claimed by applicant

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

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

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

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

Projected Publication Date: 08/18/2011

Non-Publication Request: No

Early Publication Request: No
Title

Digital Amplification

Preliminary Class

435

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The applicant has been granted a license under 35 U.S.C. 184, if the phrase "IF REQUIRED, FOREIGN FILING LICENSE GRANTED" followed by a date appears on this form. Such licenses are issued in all applications where the conditions for issuance of a license have been met, regardless of whether or not a license may be required as set forth in 37 CFR 5.15. The scope and limitations of this license are set forth in 37 CFR 5.15(a) unless an earlier license has been issued under 37 CFR 5.15(b). The license is subject to revocation upon written notification. The date indicated is the effective date of the license, unless an earlier license of similar scope has been granted under 37 CFR 5.13 or 5.14.

This license is to be retained by the licensee and may be used at any time on or after the effective date thereof unless it is revoked. This license is automatically transferred to any related applications(s) filed under 37 CFR 1.53(d). This license is not retroactive.

The grant of a license does not in any way lessen the responsibility of a licensee for the security of the subject matter as imposed by any Government contract or the provisions of existing laws relating to espionage and the national security or the export of technical data. Licensees should apprise themselves of current regulations especially with respect to certain countries, of other agencies, particularly the Office of Defense Trade Controls, Department of State (with respect to Arms, Munitions and Implements of War (22 CFR 121-128)); the Bureau of Industry and Security, Department of Commerce (15 CFR parts 730-774); the Office of Foreign AssetsControl, Department of Treasury (31 CFR Parts 500+) and the Department of Energy.

NOT GRANTED

No license under 35 U.S.C. 184 has been granted at this time, if the phrase "IF REQUIRED, FOREIGN FILING LICENSE GRANTED" DOES NOT appear on this form. Applicant may still petition for a license under 37 CFR 5.12, if a license is desired before the expiration of 6 months from the filing date of the application. If 6 months has lapsed from the filing date of this application and the licensee has not received any indication of a secrecy order under 35 U.S.C. 181, the licensee may foreign file the application pursuant to 37 CFR 5.15(b).

PATENT APPLICATION FEE DETERMINATION RECORD

Substitute for Form PTO-875

Application or Docket Number: 13/071,105

APPLICATION AS FILED - PART I

FOR	(Column 1) NUMBER FILED	(Column 2) NUMBER EXTRA	SMALL ENTITY RATE($)	SMALL ENTITY FEE($)	OR	OTHER THAN SMALL ENTITY RATE($)	OTHER THAN SMALL ENTITY FEE($)
BASIC FEE (37 CFR 1.16(a), (b), or (c))	N/A	N/A	N/A			N/A	330
SEARCH FEE (37 CFR 1.16(k), (i), or (m))	N/A	N/A	N/A			N/A	540
EXAMINATION FEE (37 CFR 1.16(o), (p), or (q))	N/A	N/A	N/A			N/A	220
TOTAL CLAIMS (37 CFR 1.16(i))	48 minus 20=	* 28			OR	x 52 =	1456
INDEPENDENT CLAIMS (37 CFR 1.16(h))	5 minus 3 =	* 2				x 220 =	440
APPLICATION SIZE FEE (37 CFR 1.16(s))	If the specification and drawings exceed 100 sheets of paper, the application size fee due is $270 ($135 for small entity) for each additional 50 sheets or fraction thereof. See 35 U.S.C. 41(a)(1)(G) and 37 CFR 1.16(s).						0.00
MULTIPLE DEPENDENT CLAIM PRESENT (37 CFR 1.16(j))							0.00
* If the difference in column 1 is less than zero, enter "0" in column 2.			TOTAL			TOTAL	2986

APPLICATION AS AMENDED - PART II

		(Column 1) CLAIMS REMAINING AFTER AMENDMENT		(Column 2) HIGHEST NUMBER PREVIOUSLY PAID FOR	(Column 3) PRESENT EXTRA	SMALL ENTITY RATE($)	SMALL ENTITY ADDITIONAL FEE($)	OR	OTHER THAN SMALL ENTITY RATE($)	OTHER THAN SMALL ENTITY ADDITIONAL FEE($)
AMENDMENT A	Total (37 CFR 1.16(i))	*	Minus	**	=	x =		OR	x =	
	Independent (37 CFR 1.16(h))	*	Minus	***	=	x =		OR	x =	
	Application Size Fee (37 CFR 1.16(s))									
	FIRST PRESENTATION OF MULTIPLE DEPENDENT CLAIM (37 CFR 1.16(j))							OR		
						TOTAL ADD'L FEE		OR	TOTAL ADD'L FEE	

		(Column 1) CLAIMS REMAINING AFTER AMENDMENT		(Column 2) HIGHEST NUMBER PREVIOUSLY PAID FOR	(Column 3) PRESENT EXTRA	RATE($)	ADDITIONAL FEE($)	OR	RATE($)	ADDITIONAL FEE($)
AMENDMENT B	Total (37 CFR 1.16(i))	*	Minus	**	=	x =		OR	x =	
	Independent (37 CFR 1.16(h))	*	Minus	***	=	x =		OR	x =	
	Application Size Fee (37 CFR 1.16(s))									
	FIRST PRESENTATION OF MULTIPLE DEPENDENT CLAIM (37 CFR 1.16(j))							OR		
						TOTAL ADD'L FEE		OR	TOTAL ADD'L FEE	

* If the entry in column 1 is less than the entry in column 2, write "0" in column 3.

** If the "Highest Number Previously Paid For" IN THIS SPACE is less than 20, enter "20".

*** If the "Highest Number Previously Paid For" IN THIS SPACE is less than 3, enter "3".

The "Highest Number Previously Paid For" (Total or Independent) is the highest found in the appropriate box in column 1.

UTILITY PATENT

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

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

For: DIGITAL AMPLIFICATION

RESPONSE TO NOTICE TO FILE MISSING PARTS OF NONPROVISIONAL APPLICATION

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

Dear Sir:

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

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

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

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

Respectfully submitted,

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

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

Dated: July 8, 2011

Electronic Patent Application Fee Transmittal

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

Filed as Large Entity

Utility under 35 USC 111(a) Filing Fees

Description	Fee Code	Quantity	Amount	Sub-Total in USD($)
Basic Filing:				
Pages:				
Claims:				
Claims in excess of 20	1202	28	52	1456
Independent claims in excess of 3	1201	2	220	440
Miscellaneous-Filing:				
Petition:				
Patent-Appeals-and-Interference:				
Post-Allowance-and-Post-Issuance:				

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

Electronic Acknowledgement Receipt

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

Payment information:

Submitted with Payment	yes
Payment Type	Deposit Account
Payment was successfully received in RAM	$1896
RAM confirmation Number	11335
Deposit Account	190733
Authorized User	
The Director of the USPTO is hereby authorized to charge indicated fees and credit any overpayment as follows:	
Charge any Additional Fees required under 37 C.F.R. Section 1.21 (Miscellaneous fees and charges)	

File Listing:

Document Number	Document Description	File Name	File Size(Bytes)/ Message Digest	Multi Part /.zip	Pages (if appl.)
1	Applicant Response to Pre-Exam Formalities Notice	001107_00866_Response_to_Notice_to_File_Missing_Parts_07_08_2011.pdf	69225 66f3abdce50127e06f04ee09626a2f03c50303b1	no	2
Warnings:					
Information:					
2	Fee Worksheet (SB06)	fee-info.pdf	31711 187f04b1ad78c83f4eb09261dd87d763eee412d8	no	2
Warnings:					
Information:					
		Total Files Size (in bytes):	100936		

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

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

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

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

UNITED STATES PATENT AND TRADEMARK OFFICE

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

APPLICATION NUMBER	FILING or 371(c) DATE	GRP ART UNIT	FIL FEE REC'D	ATTY.DOCKET.NO	TOT CLAIMS	IND CLAIMS
13/071,105	03/24/2011		1090	001107.00866	48	5

CONFIRMATION NO. 3361

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

FILING RECEIPT

OC000000047240067

Date Mailed: 05/09/2011

Receipt is acknowledged of this non-provisional patent application. The application will be taken up for examination in due course. Applicant will be notified as to the results of the examination. Any correspondence concerning the application must include the following identification information: the U.S. APPLICATION NUMBER, FILING DATE, NAME OF APPLICANT, and TITLE OF INVENTION. Fees transmitted by check or draft are subject to collection. Please verify the accuracy of the data presented on this receipt. **If an error is noted on this Filing Receipt, please submit a written request for a Filing Receipt Correction. Please provide a copy of this Filing Receipt with the changes noted thereon. If you received a "Notice to File Missing Parts" for this application, please submit any corrections to this Filing Receipt with your reply to the Notice. When the USPTO processes the reply to the Notice, the USPTO will generate another Filing Receipt incorporating the requested corrections**

Applicant(s)

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

Assignment For Published Patent Application

THE JOHNS HOPKINS UNIVERSITY, Baltimore, MD

Power of Attorney:

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

Domestic Priority data as claimed by applicant

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

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

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

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

Projected Publication Date: 08/18/2011

Non-Publication Request: No

Early Publication Request: No
Title

Digital Amplification

Preliminary Class

PROTECTING YOUR INVENTION OUTSIDE THE UNITED STATES

Since the rights granted by a U.S. patent extend only throughout the territory of the United States and have no effect in a foreign country, an inventor who wishes patent protection in another country must apply for a patent in a specific country or in regional patent offices. Applicants may wish to consider the filing of an international application under the Patent Cooperation Treaty (PCT). An International (PCT) application generally has the same effect as a regular national patent application in each PCT-member country. The PCT process **simplifies** the filing of patent applications on the same invention in member countries, but **does not result** in a grant of "an international patent" and does not eliminate the need of applicants to file additional documents and fees in countries where patent protection is desired.

Almost every country has its own patent law, and a person desiring a patent in a particular country must make an application for patent in that country in accordance with its particular laws. Since the laws of many countries differ in various respects from the patent law of the United States, applicants are advised to seek guidance from specific foreign countries to ensure that patent rights are not lost prematurely.

Applicants also are advised that in the case of inventions made in the United States, the Director of the USPTO must issue a license before applicants can apply for a patent in a foreign country. The filing of a U.S. patent application serves as a request for a foreign filing license. The application's filing receipt contains further information and guidance as to the status of applicant's license for foreign filing.

Applicants may wish to consult the USPTO booklet, "General Information Concerning Patents" (specifically, the section entitled "Treaties and Foreign Patents") for more information on timeframes and deadlines for filing foreign patent applications. The guide is available either by contacting the USPTO Contact Center at 800-786-9199, or it can be viewed on the USPTO website at http://www.uspto.gov/web/offices/pac/doc/general/index.html.

For information on preventing theft of your intellectual property (patents, trademarks and copyrights), you may wish to consult the U.S. Government website, http://www.stopfakes.gov. Part of a Department of Commerce initiative, this website includes self-help "toolkits" giving innovators guidance on how to protect intellectual property in specific countries such as China, Korea and Mexico. For questions regarding patent enforcement issues, applicants may call the U.S. Government hotline at 1-866-999-HALT (1-866-999-4158).

LICENSE FOR FOREIGN FILING UNDER

Title 35, United States Code, Section 184

Title 37, Code of Federal Regulations, 5.11 & 5.15

GRANTED

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NOT GRANTED

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

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

CONFIRMATION NO. 3361

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

FORMALITIES LETTER

OC000000047240068

Date Mailed: 05/09/2011

NOTICE TO FILE MISSING PARTS OF NONPROVISIONAL APPLICATION

FILED UNDER 37 CFR 1.53(b)

Filing Date Granted

Items Required To Avoid Abandonment:

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

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

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

SUMMARY OF FEES DUE:

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

Replies should be mailed to:

Mail Stop Missing Parts
Commissioner for Patents
P.O. Box 1450
Alexandria VA 22313-1450

Registered users of EFS-Web may alternatively submit their reply to this notice via EFS-Web.
https://sportal.uspto.gov/authenticate/AuthenticateUserLocalEPF.html

For more information about EFS-Web please call the USPTO Electronic Business Center at **1-866-217-9197** or visit our website at http://www.uspto.gov/ebc.

If you are not using EFS-Web to submit your reply, you must include a copy of this notice.

/mhteklu/

Office of Data Management, Application Assistance Unit (571) 272-4000, or (571) 272-4200, or 1-888-786-0101

PATENT APPLICATION FEE DETERMINATION RECORD Substitute for Form PTO-875	Application or Docket Number 13/071,105

APPLICATION AS FILED - PART I

	(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))	48 minus 20=	* 28			OR	x 52 =	1456
INDEPENDENT CLAIMS (37 CFR 1.16(h))	5 minus 3 =	* 2				x 220 =	440
APPLICATION SIZE FEE (37 CFR 1.16(s))	If the specification and drawings exceed 100 sheets of paper, the application size fee due is $270 ($135 for small entity) for each additional 50 sheets or fraction thereof. See 35 U.S.C. 41(a)(1)(G) and 37 CFR 1.16(s).						0.00
MULTIPLE DEPENDENT CLAIM PRESENT (37 CFR 1.16(j))							0.00
* If the difference in column 1 is less than zero, enter "0" in column 2.			TOTAL			TOTAL	2986

APPLICATION AS AMENDED - PART II

AMENDMENT A		(Column 1) CLAIMS REMAINING AFTER AMENDMENT		(Column 2) HIGHEST NUMBER PREVIOUSLY PAID FOR	(Column 3) PRESENT EXTRA	SMALL ENTITY RATE($)	SMALL ENTITY ADDITIONAL FEE($)	OR	OTHER THAN SMALL ENTITY RATE($)	OTHER THAN SMALL ENTITY ADDITIONAL FEE($)
	Total (37 CFR 1.16(i))	*	Minus	**	=	x =		OR	x =	
	Independent (37 CFR 1.16(h))	*	Minus	***	=	x =		OR	x =	
	Application Size Fee (37 CFR 1.16(s))									
	FIRST PRESENTATION OF MULTIPLE DEPENDENT CLAIM (37 CFR 1.16(j))							OR		
						TOTAL ADD'L FEE		OR	TOTAL ADD'L FEE	

AMENDMENT B		(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 found in the appropriate box in column 1.