(19) Europäisches Patentamt
European Patent Office
Office européen des brevets

(11) EP 1 230 387 B1

(12) **EUROPEAN PATENT SPECIFICATION**

(45) Date of publication and mention of the grant of the patent:
16.08.2006 Bulletin 2006/33

(21) Application number: **00970925.4**

(22) Date of filing: **13.10.2000**

(51) Int Cl.:
C12Q 1/68 *(2006.01)* ***C12P 19/34*** *(2006.01)*
C07H 21/04 *(2006.01)* ***C07H 21/00*** *(2006.01)*

(86) International application number:
PCT/US2000/028515

(87) International publication number:
WO 2001/031062 (03.05.2001 Gazette 2001/18)

(54) **ASSAYS FOR SHORT SEQUENCE VARIANTS**

NACHWEISVERFAHREN FÜR KURZE SEQUENZVARIANTEN

MÉTHODE DE DÉTECTION DE VARIANTS DE SÉQUENCES COURTES

(84) Designated Contracting States:
AT BE CH CY DE DK ES FI FR GB GR IE IT LI LU MC NL PT SE

(30) Priority: **22.10.1999 US 161096 P**

(43) Date of publication of application:
14.08.2002 Bulletin 2002/33

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(56) References cited:
WO-A-99/40226
US-A- 5 210 015
US-A- 5 487 972
US-A- 5 925 517

- **MARRAS S A E ET AL: "Multiplex detection of single-nucleotide variations using molecular beacons" GENETIC ANALYSIS: BIOMOLECULAR ENGINEERING, ELSEVIER SCIENCE PUBLISHING, US, vol. 14, no. 5-6, February 1999 (1999-02), pages 151-156, XP004158697 ISSN: 1050-3862**
- **PIATEK A S ET AL: "MOLECULAR BEACON SEQUENCE ANALYSIS FOR DETECTING DRUG RESISTANCE IN MYCOBACTERIUM TUBERCULOSIS" NATURE BIOTECHNOLOGY, NATURE PUBLISHING, US, vol. 16, April 1998 (1998-04), pages 359-363, XP000891876 ISSN: 1087-0156**
- **KOSTRIKIS L G ET AL: "MOLECULAR BEACONS: SPECTRAL GENOTYPING OF HUMAN ALLELES" SCIENCE, AMERICAN ASSOCIATION FOR THE ADVANCEMENT OF SCIENCE,, US, vol. 279, no. 20, 20 February 1998 (1998-02-20), pages 1228-1229, XP002942660 ISSN: 0036-8075**
- **VET J A M ET AL: "Multiplex detection of four pathogenic retroviruses using molecular beacons" PROCEEDINGS OF THE NATIONAL ACADEMY OF SCIENCES OF USA, NATIONAL ACADEMY OF SCIENCE. WASHINGTON, US, vol. 96, 1999, pages 6394-6399, XP002145609 ISSN: 0027-8424**
- **TYAGI S ET AL: "MOLECULAR BEACONS: PROBES THAT FLUORESCE UPON HYBRIDIZATION" NATURE BIOTECHNOLOGY, NATURE PUBLISHING, US, vol. 14, 1 March 1996 (1996-03-01), pages 303-308, XP000196024 ISSN: 1087-0156**

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Description

Background of the Invention

[0001] Serious infections require early treatment with effective antibiotics [Mandell et al. (1995) Principles and practice of infectious diseases. 4th edition. Churchill Livingstone, New York]. Because definitive identification of most bacteria, using a combination of culture, morphological and biochemical tests, usually requires several days to complete, most infectious diseases are treated empirically with broad spectrum antibiotics [Weinstein (1968) *Pediatr. Clin. North Am.* 15:141-156; Moellering (1974) *In: Seminar on Gram-Negative Infections.* St. Louis 1974:5; Cassiere et al. (1998) *Dis. Mon.* 44:613-675]. The emergence of multidrug-resistant bacteria has reduced the effectiveness of this practice. It has become increasingly difficult to find antibiotics (or combinations of antibiotics) that are reliably effective against all of the etiologic organisms of a specific syndrome. This problem is exacerbated where an unusual microbial (e.g., bacterial, mycoplasmal, viral, or parasitic) agent results in an infectious syndrome that can be confused with one caused by one (or more) more common pathogens. Simpler and more rapid identification methods and subsequent pathogen-specific therapy are becoming increasingly important in the treatment of infectious diseases [Casadevall (1996) *Clin Infect Dis.* 23:790-794]. U.S. Patent Nos. 5,487,972, 5,538,848, and 5,925,517, published International Application No. WO 97/39008, and International Application No. PCT/US99/17145.

[0002] Differentiation among closely related sequences can be obtained in limited circumstances by utilizing a different allele-discriminating molecular beacon probe that is specific for each sequence. Marras et al. (1999) *Genetic Analysis: Biomolecular Engineering* 14: 151-156; Piatek et al. (1998) *Nature Biotechnology 16:* 359-363. Only a limited number of alleles can be analyzed, because only a limited number of fluorophores can be distinguished, the sequences of all variants must be known, and the probes must be carefully constructed to operate in allele-discriminating fashion at the same reaction conditions, for example. Differentiation under limited circumstances can be obtained with linear probes in the 5' nuclease assay. WO-A-99/40226. Each target must be probed at two allelic sites by allele-specific probes, which in combination can reveal whether a sample is homozygous or heterozygous for up to three alleles at least. Again the sequence of all variants must be known, only for a limited number of fluorophores can be used, and the probes are carefully designed to maximize specificity.

Summary of Invention

[0003] In one aspect, the invention provides a homogeneous detection assay for identifying, in a sample, a variant of a gene from among a number of possible variants of a short nucleotide sequence having a first region differing among said variants, the assay comprising:

(a) providing an aliquot of a sample suspected of containing said short nucleotide sequence;
(b) forming a reaction mixture that includes said aliquot;
(c) probing said reaction mixture with a set of molecular beacon probes for said first region but differing in their target binding sequences under hybridisation conditions such that each probe in the set is able to hybridise to more than one of the possible variants with varying degrees of complementarity, wherein different probes in the set are differently fluorescently labelled, wherein each probe in the set has a single stranded loop target binding sequence 25 - 50 nucleotides in length and arms hybrids 4 - 6 nucleotides in length said probes generating, wherein the probes generate, in said reaction mixture, detectable fluorescent signals indicative of their hybridization to said first region, and wherein the signal emitted by each probe is separably detectable;
(d) measuring the intensity of said signals; and
(e) determining the ratio of normalised fluorescence intensities emitted by all pairs of molecular beacon probes present in the reaction mixture as an indication of the presence or absence of said variant in said sample.

[0004] In another aspect, the invention provides a kit of reagents suitable for identifying one of multiple possible variants of a gene in a sample, the gene having a first region differing among said variants, said kit comprising a set of molecular beacon probes for said first region but differing in their target binding sequences, wherein different probes in the set are differently fluorescently labelled, wherein each probe in the set has a single-stranded loop target binding sequence 25 - 50 nucleotides in length and arms hybrids 4 - 6 nucleotides in length, and wherein each probe in the set is able to hybridise to more than one of the possible variants with different degrees of complementarity, wherein said probes generate detectable fluorescent signals indicative of their hybridization to said first region, and wherein the signals emitted by each of said probes are separably detectable.

[0005] The invention also allows for the assessment of the relatedness of two nucleic acid regions when the sequence of one or both regions are unknown. Assays and kits according to this invention have applications that include mutational analysis and pathogen identification.

[0006] Labeled oligonucleotide probes for use in methods of this invention are designed such that each will bind to multiple variants, or alleles. of a particular nucleic acid sequence. We refer to probes useful in methods of this invention as "sloppy" probes. When used in combinations, two or more such probes provide a means to detect the presence of one variant from among multiple possible variants. The invention provides an inexpensive and rapid diagnostic method that is able to identify a broad range of known and unknown microorganisms (e.g., bacteria, mycoplasmas, viruses, and parasites) and is easily automated. In addition. the assay can be used to identify genetic variants associated with mammalian (e.g.. human) diseases such as cancer, autoimmune diseases, or metabolic diseases, or variations in genes of any eukaryotic organisms.

[0007] The reaction mixture can be an amplification reaction mixture. with the assay further including, after forming the amplification reaction mixture. amplifying the first region, if present in the sample. The amplification reaction mixture can be a polymerase chain reaction (PCR) amplification mixture that includes a primer pair and an appropriate DNA polymerase. The primer binding regions are relatively conserved between the gene and the variant or among variants, as among bacterial species. Amplifications other than PCR that can be used in the assays of the invention include, without limitation, Q-Beta replicase-mediated amplification, transcription and replication reactions such as nucleic acid sequence-based amplification (NASBA) and self-sustained sequence replication (3SR), ligase chain reaction (LCR) or strand displacement amplifications (SDA).

[0008] The oligonucleotide hybridization probes, or sloppy probes, used in the assays of the invention are fluorescently labeled molecular beacon probes that produce a detectable signal in a homogeneous assay, that is, without having to separate probes hybridized to target from unbound probes. The probes are dual-labeled having interactive labels consisting of a fluorophore and a quencher. such that hybridization to a target sequence or hybridization to a target sequence and reaction in the assay results in a detectable fluorescence signal. The probes can be added to the amplification reaction mixture before, during, or after the amplification. The molecular beacon probes have pairs of arms that are complementary to one another and quench one another when they are hybridized to each other but not when hybridized to target [Morrison and Stols (1993) Biochemistry 32: 309-3104].

[0009] Dual-labeled hairpin probes are described in U.S. Patent No. 5,925,517, published International Application No. WO97/39008.

[0010] These hairpin probes contain a target binding sequence flanked by a pair of arms complementary to one another. They can be DNA, RNA, or PNA, or a combination of all three nucleic acids. Furthermore, they can contain modified nucleotides and modified internucleotide linkages. They can have a first fluorophore on one arm and a second fluorophore on the other arm. wherein the absorption spectrum of the second fluorophore substantially overlaps the emission spectrum of the first fluorophore. The probes need not be cleavable during amplification. The "molecular beacon probes" have a fluorophore on one arm and a quencher on the other arm such that the probes are dark when free in solution. They can also be wavelength-shifting molecular beacon probes with for example, multiple fluorophores on one arm that interact by fluorescence resonance energy transfer (FRET), and a quencher on the other arm. The target binding sequences is 25 to 50 nucleotides in length, and the hybridizing arms are to 6 (e.g., 5 or 6) nucleotides in length. Molecular beacon probes can be tethered to primers, as described in Whitcombe et al. (1999), Nature Biotechnology 17:904-807.

[0011] Assays according to this invention can be chip-based, that is. utilizing sloppy probes immobilized at known locations on a solid surface. Conventional chip-based methods utilize immobilized probes that are specific for particular sequences. Replacing at least some of the conventional immobilized probes with sloppy molecular beacon probes and deriving sequence information from the pattern of hybridization to multiple probes as taught herein permits a reduction in the number of required immobilized probes by as much as an order of magnitude, in some assays by as much as two orders of magnitude.

[0012] The variants detected by the assays of the invention can contain a second region not overlapping the first region. If so, the assay can include the following additional steps: (f) probing nucleic acid molecules in the reaction mixture with a supplemental oligonucleotide hybridization probe having a target binding sequence hybridizable to the second region. if present, and not hybridizable to the first region. the supplemental probe being capable of generating in the assay reaction mixture a detectable signal indicative of its hybridization to the second region, wherein the signals emitted by each of the at least two probes and the supplemental probe are separably detectable; (g) measuring the intensity of the signal emitted by the supplemental probe; and (h) additionally determining at least one ratio of fluorescence intensities emitted by the supplemental probe and one of the at least two probes as part of the indication of the presence or absence of the variant in the sample. The supplemental probe is specific for the second region, that is, of conventional design.

[0013] The variants to be detected can be variants of eukaryotic genes. including mammalian genes. Thus the variant can be a somatic mutant of a mammalian gene. e.g., an oncogene (such as a *ras* mutation. Alternatively, it can be a mammalian allele or somatic mutant (e.g., an allele of the globin gene) associated with a metabolic disease (e.g., sickle cell anemia, thalassemia, cystic fibrosis. Gaucher disease) or an allele or somatic-mutant (e.g., a major histocompatibility complex (MHC) gene, a immunoglobulin (Ig) gene, or a T cell receptor (TCR) gene) associated with an autoimmune disease (e.g., rheumatoid arthritis (R.A), multiple sclerosis, insulin-dependent diabetes mellitus (IDDM), muscular dys-

trophy (MD), myasthenia gravis (MG), or systemic lupus erythematosus (SLE)).

[0014] Alternatively, the gene can be a microbial (e.g., bacterial, viral, or parasitic) allele. An example of an appropriate family of genes is the family of ribosomal RNA (rRNA) genes that contain highly conserved DNA sequences interspaced with variable species-specific regions [Woese (1987) *Microbiol. Rev.* 51:221-271]. DNA from a wide variety of microorganisms can be amplified using a single set of primers to conserved regions and species determination subsequently performed by analyzing species-specific sequences [Pace (1997) *Science,* 276:734-740]. A bacterial gene can be a *Mycobacterium* gene. Alleles of the mycobacterial 16S rRNA genes [Kirschner et al. (1993) *J. Clin. Microbiol.* 31: 2882-2889; Vaneechoutte et al. (1993) *J. Clin. Microbiol.* 31:2061-2065; Kox et al. (1995) *J. Clin. Microbiol.* 33:3225-3233) can be used for mycobacterial species identification. In addition. rRNA alleles can be used to define species of *Gonococci* and *Chlanrydia* [Kluytznaas et al. (1991) J. *Clin. Microbiol.* 292685-2689; Iwen et al. (1995) *J. Clin. Microbiol.* 33: 2587-2591]. Other conserved genes, such as *rpoB* or heat shock proteins, can also be used for bacterial species identification [Telenti et al. (1993) *Clin. Microbiol.* 31:175-178; Mollet et al. (1997) *Mol. Microbiol.* 26:1005-1011].

[0015] As explained above, the invention also includes kits of reagents containing combinations of the above-mentioned probes for detecting any of the above mentioned genetic variants in a sample. Embodiments of kits may include chips having sloppy molecular beacon probes, immobilized at predetermined locations. The chips may also include supplemental probes or other conventional probes.

[0016] As used herein, "variants of a gene" are understood to include allelic variants and somatic mutants of a gene, and species-specific, subspecies-specific, and strain-specific versions of a gene. The "first region" of the variants, can differ from the equivalent region of the gene or other variations thereof by one or more nucleotide substitutions, one or more nucleotide additions, or one or more nucleotide deletions. While the set of molecule beacon probes can be capable of binding to the region of the gene equivalent to the first region of the variant, it is not required that they do so provided that under the hybridisation conditions of the assay, each probe in the set is able to hybridise to more than one of the possible variants with varying degrees of complementarity. It is understood that the term "variant" includes the complement of the relevant sequence.

[0017] As used herein, a "sample" can be one or more cells (eukaryotic or prokaryotic) a tissue, a cell or tissue lysate, a bodily fluid, excreta (e.g., urine or feces), a microbial colony or plaque, a solution containing nucleic acid (e.g., DNA, cDNA, or RNA) purified or semi-purified from any of the above sources, amplification (PCR or other) products either in the amplification reaction mixture or isolated from it.

[0018] As used herein, a "reaction mixture" is a solution in which probing with the at least two oligonucleotide hybridization probes (and optionally a supplemental oligonucleotide hybridization probe), as a step in the assay of the invention, is performed. The reaction mixture can be, for example, a solution composed of the "aliquot of the sample" only, or it can contain other components, e.g., buffer components. The reaction mixture can be an amplification (e.g., a PCR) reaction mixture and can contain the components necessary for an amplification reaction. e.g.. nucleotides or deoxynucleotides, amplification primers or promoters, and enzymes (e.g., a DNA polymerase). If the assay includes amplification, the oligonucleotide hybridization probes can be added to the amplification reaction mixture before, during, or after amplification.

[0019] Unless otherwise defined, all technical and scientific terms used herein have the same meaning as commonly understood by one of ordinary skill in the art to which this invention pertains. In case of conflict, the present document, including definitions, will control. Preferred methods and materials are described below by way of example only.

[0020] The materials, methods, and examples are illustrative only and not intended to be limiting.

[0021] Other features and advantages of the invention, e.g., methods of identifying bacterial species in samples, will be apparent from the following description, from the drawings and from the claims.

Brief Description of the Drawings

[0022]

FIG. 1 is a depiction of the physical mechanism by which molecular beacons function as detection probes.

FIG. 2 is a line graph showing the increase in number, over time, of amplicons in a series of PCR amplifications as detected by molecular beacon probes.

FIGS. 3A and 3B are line graphs showing the increase in number, over time, of amplicons in a series of PCR amplifications as detected by molecular beacon probes with target hybridizing sequences fully complementary to a target sequence in *M. tuberculosis* (FIG. 3A) and *M. xenopie* (FIG. 3B). Each line represents the data obtained from a PCR assay containing a template from a particular species of *Mycobacteria.*

FIGS. 4A and 4B are line graphs showing the increase, over time, of fluorescence from a pair of molecular beacon

probes in a series of PCR assays containing a range of concentrations of template, and FIG. 4C presents the fluorescence ratios obtained from FIGS. 4A and 4B.

FIG. 5 is a bar graph showing the ratios of fluorescence intensity calculated for eight PCR assays containing template DNA from one of eight different species of Mycobacterium and four different sloppy molecular beacon probes.

Description of the Preferred Embodiments

[0023] The inventors have discovered that probes (sometimes referred to as "sloppy probes"), by virtue of their ability to bind to more than one (e.g., 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 15, 20, 30, 40, 100, or 1000) variants of a given target sequence, can be used in assays to detect the presence of one variant of a nucleic acid sequence segment of interest from among a number of possible variants or even to detect the presence of two or more variants. The probes are used in combinations of two or more in the same assay. Because they differ in target binding sequence, their relative avidities for different variants are different. For example, a first probe may bind strongly to a wild-type sequence, moderately to a first allele, weakly to a second allele and not at all to a third allele; while a second probe may bind weakly to the wild-type sequence and the first variant, and moderately to the second variant and the third variant. Additional sloppy probes will exhibit yet different binding patterns due to their different target binding sequences. Thus, fluorescence emission spectra from combinations of sloppy probes define different microbial strains or species, as well as allelic variants of genes in mammalian tissues that are associated with diseases and somatic mutations.

[0024] Assays according to this invention are described below utilizing "sloppy molecular beacon probes." Because sloppy probes reproducibly fluoresce with variable intensities after binding to different DNA sequences, combinations can be used in, for example, simple, rapid, and sensitive nucleic acid amplification reaction assays (e.g., PCR-based assays) that identify multiple pathogens in a single reaction well. It is understood, however, that the assays can be performed also on samples suspected of containing directly detectable amounts of unamplified target nucleic acids. This novel species identification assay is based on deconvolving the spectra of a set of partially hybridizing sloppy signaling sloppy molecular beacon probes, each labeled with a fluorophore that emits light with a different wavelength optimum, to generate "signature spectra" of species-specific DNA sequences.

Operation Principles of Molecular Beacon Probes

[0025] The use of conformation-dependent fluorescent probes called "molecular beacon" probes to detect short DNA sequences with single nucleotide accuracy in real-time PCR reactions has been described [Tyagi et al. (1996) *Nat. Biotechnol.* 14:303-308; Kostrikis et al. (1998) *Science.* 279: 1228-1229: Piatek et al. (1998) *Nat. Biotechnol.* 16:359-363]. With reference to FIG. 1 molecular beacon probe I is a single-stranded fluorescent nucleic acid molecule that possess a hairpin structure comprising loop 2 flanked by arms 3 and 4. Loop portion 2 serves as a probe sequence, or target binding sequence, that is complementary to a target DNA sequence. The probe sequence is embedded within short flanking arm sequences 3 and 4, which hybridize to each other to form a stem structure. In certain embodiments one arm sequence or a portion thereof may also be complementary to the target. A fluorescent moiety 5 is covalently linked to one arm, most conveniently at the free end, and a nonfluorescent quenching moiety 6 is covalently linked to the other arm, again, most conveniently at the free end. In homogenous solutions, the close proximity of the fluorophore to the quencher minimizes fluorescence, when the molecular beacon is in the stem-and-loop conformation. When the loop hybridizes to its target 7, the rigidity of the resulting probe-target helix 8 forces the arm sequences apart. The fluorophore 5 is thereby separated from the quencher 6, permitting the fluorophore to fluoresce brightly when excited by light of an appropriate wavelength.

[0026] Molecular beacon probes have a number of advantages for use in assays of this invention. Molecular beacons are able to detect amplicons as they are synthesized during amplification. In real-time PCR, for example, the fluorescence generated by molecular beacon-target hybrids can be measured, by a spectrofluorometric thermal cycler which plots the fluorescence intensity as a function of the number of PCR thermal cycles performed. FIG. 2 is a typical graph of a series of PCR reactions of an allele-dischminadng molecular beacon probe, which may be used as the supplementary probe in methods of this invention, with varying starting amounts of perfectly matched target, wild-type DNA. and varying starting amounts of mutant DNA differing by a single nucleotide to which the probe essentially does not hybridize. The data in FIG. 2 indicate that, as increasing amounts of wild-type amplicon, but not mutant amplicon, are synthesized, the fluorescence due to the molecular beacon-target hybrids also increases, thereby resulting in a characteristic fluorescent curve. Reagents can be combined in, for example, the wells of a 96-well microtiter plate, or individual reaction tubes, which are then hermetically sealed. Molecular beacons with different target binding sequence can be labeled with differently colored fluorophores and used simultaneously in the same assay. Because the probes are dark when not hybridized, background fluorescence is low and separation of signals from multiple probes is improved. Amplification, molecular beacon hybridization, and analysis are all performed simultaneously.

Sloppy Molecular Beacons

[0027] Molecular beacon probes useful in the methods of this invention will hybridize to more than one variant and are herein designated "sloppy" molecular beacon probes. The probe sequences (i.e., the loop or the target hybridizing sequence) of sloppy molecular beacons are longer than the probe sequence of molecular beacon probes that hybridize only to perfectly matched target sequences.

[0028] Persons skilled in the art can readily prepare sloppy probes by minimal trial and error. For random coil (or "linear") probes such as TaqMan™ probes described in U.S. Patent No. 5,487,972, the length of the probe region complementary to intended targets is increased sufficiently that the probe binds not only to perfectly matched targets but also to targets differing, as need arises, by one or several nucleotides. For molecular beacon probes, the length of the probe region is increased but the length of the arms hybrid is kept short. We have found loop sequences in the range of 25 to 50 nucleotides in length and arms hybrids in the range of 4 to 6 nucleotides in length to be generally satisfactory and to provide an excellent starting point for probe design.

[0029] Sloppy molecular beacons can readily be designed to hybridize and fluoresce strongly to both perfectly complementary targets and to a wide range of mismatched targets at typical annealing temperatures, 40°C to 55°C, of PCR assays. We performed real-time PCR assays containing sloppy molecular beacons using different target amplicons with varying degrees of complementarity to the probe region. FIGS. 3A and 3B show the results of real-time PCR assays using either a fluorescein (FAM)-labeled molecular beacon with a 45-nucleotide probe region that was perfectly complementary to a species-specific hypervariable region of the *M. tuberculosis* 16S rRNA gene (FIG. 3A) or a tetrachlorofluorescein (TET)-labeled molecular beacon with a 45-nucleotide probe region that was perfectly complementary to a species-specific hypervariable region of the *M. xenopie* 16S rRNA gene (FIG. 3B). As expected, these sloppy molecular beacons fluoresced strongly in the presence of perfectly complementary target during real-time PCR. However, they also demonstrated a wide range of fluorescence intensities when used in the presence of seven other partially non-complementary amplicons from other mycobacterial species. Gel electrophoresis demonstrated that the differences in fluorescence intensity were not due to differences in amplicon concentration, but rather, to the proportion of amplicons to which molecular beacons are bound at the annealing temperature of the assay. The target amplicons differed from *M. tuberculosis* or *M. xenopie* at 2-8 base pairs (4%-18% non-complementary with the probe region).

[0030] Every molecular beacon-target hybrid with a unique melting temperature will have a corresponding unique signal intensity at a defined temperature and concentration of probe and amplicon. Thus, a limited number of sloppy probes could be used as probes to identify many different possible target sequences in a real-time PCR reaction, if it were possible to control for differences in amplicon concentration, and to control for well-to-well variations in background fluorescence. Fluorescence intensity during real-time PCR is influenced by the concentrations of probes and amplicons present in the reaction. The concentration of probes can be controlled experimentally, but different amounts of amplicon are generated with each PCR reaction. In order to obtain measurements that are independent of target molecule concentration, assays according to this invention utilize the ratios of fluorescence of all pairs of probes present in the reaction mixture. In calculating the ratios, the concentration parameters cancel out. As long as the same master mix of probes is used, the fluorescence ratios of pairs of different sloppy molecular beacon probes hybridizing to the same target amplicon varied by less then 10% over a 10,000-fold change in target concentration.

[0031] Referring to FIGS. 4A, 4B and 4C, a master mix containing two sloppy molecular beacon probes was prepared. One probe was labeled with TET. The other probe was labeled with FAM. The master mix was used in a series of PCR amplifications wherein eight amounts of starting concentration of target were used, varying from a maximum of 10 ng to a minimum of 1 pg. The TET-labeled probe was fully complementary to the target. The loop sequence of the FAM-labeled probe was fully complementary to a neighboring region of the target. FIG. 4A shows the fluorescence intensities obtained for the TET-labeled probe as a function of the number of PCR cycles performed. FIG. 4B shows the fluorescence intensities obtained for the FAM-labeled probe as a function of the number of PCR cycles performed. FIG. 4C is a plot of the ratios of TET intensities to FAM intensities. The non-variance of the ratios is apparent. In contrast, the fluorescence ratios of two sloppy molecular beacons hybridizing to two different target amplicons can vary by over 600%.

[0032] Mathematical models of molecular beacon-target interactions support these experimental observations. The inventors have determined mathematically that fluorescence has a linear relationship to target concentration, when the target concentration is sufficiently high. By analyzing fluorescence ratios (instead of individual fluorescence intensities), once the linear (or plateau) phase of PCR has been reached, the concentration dependence is canceled out (FIG. 4C). This enables a concentration independent measure of molecular beacon hybridization. Concentration effects are similarly eliminated in assays employing no amplification, with amplification other than PCR. and with other sloppy probes.

Sloppy Molecular Beacons Used in Combination to Identify Mycobacterial Species

[0033] An assay according to this invention generates fluorescence "fingerprints" of short DNA sequences that uniquely identify a particular DNA, even if the actual DNA sequence is not known. An example will be described utilizing four

sloppy molecular beacon probes used to assay eight different mycobacterial species. Table 1 gives the sequences of a hypervariable species-specific region of the mycobacterial 16S rRNA gene for several species, showing nucleotides that differ from M. tuberculosis (M.tb).

Table 1. Species-specific DNA sequences within the hypervariable region A of selected mycobacterial 16S rRNA genes

CGG ATA GG- ACCA CGG GAT TCA TG TCT- TGT GGT GGA AAG CGC	*M.tb complex*
. . . . . T .AA . . C . . . . . - .C.	*M. avium*
. . . . . T TTA .GC . . . . . - .TA	*M. intracellulare*
. . . . . T TTA . . C . . . . . - .T.	*M. intracellulare serovar 18*
. . A . . . . . T TTA . . C . . . . . - .T.	*M. intracellulare serovar 7*
. . . . . TT .AA .GC . . . . . - .T. . . . . T	*M. leprae*
. . . . . TT .GC . . . . . C . . -	*M. simiae*
. . A . . . . . T . . A .GC . . . . . C . . -	*M. heidelbergense*
. . . . . T .TC .GC . . . . . C . . A G.A	*M. intermedium*
. . A . . . . . C . . A .GC . . . . . C . . - . . G	*M. malmoense*
. . . . . C . . A .GC . . . . . C . . - . . G	*M. szulgai*
. . . . . T .AA .GC . . . . . C . . T	*M. haemophilum*
. . . . . T . - . . . . . A.C . . . . . .T . -	*M. genavense*
. . A . . . . . A . . . C A . . . . . C -	*M. gordonae I*
. . A . . . . . A . A.C A . . . . . C -	*M. gordonae II*
. . . . . C -	*M. asiaticum*
. . . . . T . . . . . C -	*M. marinum*
. . . . . .AT .TC . . . . . GTG-	*M. triviale*
. . . . . TTC TGC . . . . . .GG- G	*M. xenopi*

[0034] We prepared four differently labeled sloppy molecular beacon probes, each of which had a target-hybridizing sequence (loop) that was complementary to one species. The sequences of the probes, including their 5-nucleotide arms, are shown in Table 2. The quencher in each case was DABCYL.

Table 2. Probe Sequences

[0035]

M. AVIUM-TMR:
5' CGACG- CGG ATA GGA CCT CAA GAC GCA TGTCTT CTG GTG GAA AGC T -CGTCG

M. XENOPI-TET:
5' CGACG- CGG ATA GGA CCA TTC TGC GCA TGT GGG GTG GTG GAA AGC GT -CGTCG

M. tuberculosis.) -FAM:
5' CGATCGG- CGG ATA GGA CCA CGG GAT GCA TGT CTT GTG GTG GAA AGC GCT -CCGATCG

M. FLAVESCENS-RhD:
5' CGACG- CGA ATA TTC CCT ATT GGT CGC ATGGCC TGG TAG GGG AAA GCG CT -CGTCG

[0036] A master mix of the four sloppy probes was tested against eight different species. All six possible fluorescence

ratios were determined, as described above in connection with FIG. 4C. A segment of 16S DNA from eight different mycobacterial species, each in a separate tube, was then amplified in the presence of all four sloppy molecular beacons. All of the PCR reactions used the same set of primers that hybridized to conserved sequences flanking the hypervariable sequence that identifies each species. Fluorescence was measured in real-time. In these experiments, the ABI 7700 spectrofluorometric thermal cycler derives an emission spectra for each fluorophore from the combined emissions of all four fluorophores in the reaction well. These data were then used to calculate fluorescence intensity for each sloppy molecular beacon. In order to normalize for differences in background fluorescence between each reaction well, the initial fluorescence in each well was subtracted from the final measured fluorescence at the end of the PCR reaction in the same well.

[0037] A ratio of fluorescence values between each two molecular beacons present in the tube was generated, resulting in six fluorescence ratios per tube. A unique set of fluorescence ratios (fluorescence fingerprints) was thus derived for the 16S DNA sequence for each species tested (FIG. 5). The experiments were then repeated three times. FIG. 5 shows the mean ratio from the three repeats. It also includes error bars showing the data spread from the mean. The negativity of some ratios is an artifact of the ABI fluorescence calculations and does not imply the disappearance of fluorescence. The negative values can be used accurately to calculate fluorescence ratios.

[0038] Each DNA sequence generated a characteristic fluorescence fingerprint. Importantly, the diversity of fluorescence ratios relates to the degree of sequence diversity among the target amplicons. The ratios were most similar for *M. tuberculosis* and *M marinum,* whose sequences in the target region only differ from one another at two nucleotide positions (Table 1). The ratios obtained for less closely related species differed markedly. Subsequent experiments confirmed the accuracy of this pattern. Despite the similarity of *M. tuberculosis* and *M. marinum,* none of the triplicate RHD/FAM ratios overlapped between these two species, demonstrating that the ratios were unique to each species. For *M. tuberculosis,* the RHD/FAM ratios were -0.23 +/- 0.01, while for *M. marinum* the RHD/FAM ratios were -0.29 +/- 0.005. All of the other species also had at least one fluorescence ratio where none of the triplicate measurements overlapped with the ratio of any other species.

[0039] A potential problem in the assays of the invention arises when more than one allele or allelic amplicon is present in the same reaction well, making deconvolution of spectra very difficult. Examples of such situations include: (a) analyses of samples (e.g., tissue, blood, excretions, or secretions) that contain more than one species, subspecies, or strain of a particular pathogenic micro-organism (e.g., mycobacteria); and (b) analysis of DNA from eukaryotic cells that are heterozygous for the variant sequence of interest. Where mammalian tissues, blood, or other bodily fluids are being tested, the chance of infection by related bacterial species is low: hence, only one variant of a gene is likely to occur in a sample. Where bacterial gene variants are encountered in a sample, this problem can be obviated by using DNA from individual colonies as a source of template.

[0040] An alternative solution to the problem is to perform the PCR amplifications in multiple vessels (e.g., wells of a microtiter plate) in each of which there is less than one genome-equivalent of the DNA. If there is a single variant present in the original sample, in vessels giving a positive result (i.e., showing significant fluorescence ratios), there should be monophasic distribution of values, i.e., all the vessels should yield a similar value within the precision of the method. If there are two variants in the sample, there should be a biphasic distribution of values among the positive PCR reaction vessels. In one set of vessels, the ratios will cluster around a first value indicative of one variant (e.g., a wild-type allele) and in the second set vessels, around a second value indicative of a second variant (e.g., a mutant allele). Where three variants are present, a triphasic distribution would be obtained, and so on. The content of reaction vessels giving anomalous values which do not fall into a particular group can be isolated and the amplicons sequenced to test for the presence of two or more amplicons in the wells.

[0041] The use of multiple (e.g., 3, 4, 5, 6, 8 or 10) sloppy beacon probes and calculation of all possible ratios can overcome discrimination problems due, for example, to the ratio of fluorescence intensity ratio for a single sloppy molecular beacon pair being the same for two or more candidate target sequences.

Wavelength-shifting molecular beacons

[0042] Four different sloppy molecular beacons may in some cases be insufficient to resolve a large number and variety of target sequences with high precision. The number of different sloppy molecular beacons that can be used simultaneously in the same assay well is only limited by the ability to resolve the emission spectrum of each fluorophore. One of the factors that limits the sensitivity of detection by fluorescence is that the optimal emission wavelength of most fluorophores is only a few nanometers longer than their optimal excitation wavelength (Stokes shift). As a consequence of this, a portion of the excitation light reaches the detector by processes such as scattering and reflection, contributing to a background signal that limits the sensitivity. Monochromatic light sources, such as lasers, are often used to minimize the extent to which the excitation light reaches the detector. However, this prevents the use of a large number of different fluorophores in the same solution, because these light sources excite some fluorophores very well but excite other fluorophores not as well or not at all. For example, the commonly used blue argon ion laser is suitable for exciting

fluorescein but not Texas red, because it excites Texas red only at about two percent of its optimal level.

[0043] Wavelength-shifting molecular beacons (WO 00/06778) permit more different probes to be used with a monochromatic light source. A wavelength-shifting molecular beacon probe can be excited, for example, by a blue argon ion laser, but emit strong fluorescent light in the orange, red, or near-infrared wavelengths. Wavelength-shifting molecular beacons contain a harvester fluorophore and an emitter fluorophore on one arm, plus a quencher such as DABCYL on the other arm. The harvester, the emitter, and quencher can be disposed in a number of positions with respect to each other. The harvester fluorophore is chosen to have high absorbance in the wavelength range of the available monochromatic light source. The emitter fluorophore is chosen to have high absorbance in the wavelength range of the harvester's emission Whereas the blue argon ion laser will not excite Texas red, fluorescein will. Thus, a wavelength-shifting molecular beacon with a fluorescein harvester and Texas red emitter is excited quite well by the same light source. In a hairpin conformation when not bound to target, the quencher quenches fluorescence, and wavelength-shifting molecular beacons emit little light in the emission ranges of either fluorophore. The light energy absorbed by the harvester fluorophore is efficiently channeled to the quencher moiety and is lost as heat. When the probe sequence in the loop binds to its target and the arms are forced apart and the quencher is no longer effective. In this conformation, the harvester and emitter fluorophore interact. The fluorescence of the harvester fluorophore is not restored, because its stored energy is rapidly transferred via resonance energy transfer (FRET) to the emitter fluorophore that is placed at an appropriate (FRET) distance. The emitter fluorophore then emits the received energy in its own characteristic range of emission thereby providing a large Stokes shift.

Claims

1. A homogeneous detection assay for identifying, in a sample, a variant of a gene from among a number of possible variants of a short nucleotide sequence having a first region differing among said variants, the assay comprising:

   (a) providing an aliquot of a sample suspected of containing said short nucleotide sequence;
   (b) forming a reaction mixture that includes said aliquot;
   (c) probing said reaction mixture with a set of molecular beacon probes for said first region but differing in their target binding sequences, under hybridisation conditions such that each probe in the set is able to hybridise to more than one of the possible variants with varying degrees of complementarity, wherein different probes in the set are differently fluorescently labelled, and wherein each probe in the set has a single stranded loop target binding sequence 25 - 50 nucleotides in length and arms hybrids 4 - 6 nucleotides in length, said probes generating in said reaction mixture, detectable fluorescent signals indicative of their hybridization to said first region, and wherein the signal emitted by each probe is separably detectable;
   (d) measuring the intensity of said signals; and
   (e) determining the ratio of normalised fluorescence intensities emitted by all pairs of molecular beacon probes present in the reaction mixture as an indication of the presence or absence of said variant in said sample.

2. The assay of claim 1, wherein said reaction mixture is an amplification reaction mixture containing the set of probes, the assay further comprising, after forming said amplification reaction mixture, amplifying said first region if present in the sample; optionally wherein said amplification reaction mixture is a PCR amplification mixture that includes a primer pair and a DNA polymerase.

3. The assay of claim 1 or claim 2, wherein said short oligonucleotide sequence has a length of up to fifty nucleotides or is a gene sequence, optionally a mammalian or microbial gene, e.g. a bacterial gene or a mycobacterial gene.

4. The assay of any one of claims 1 to 3, wherein said probes are immobilized at preselected locations on a solid surface.

5. The assay of any one of claims 1 to 4, wherein:

   (a) said variant is an oncogene; or
   (b) said variant is associated with a metabolic disease; or
   (c) said variant is associated with an autoimmune disease.

6. A kit of reagents suitable for identifying one of multiple possible variants of a gene in a sample, the gene having a first region differing among said variants, said kit comprising a set of molecular beacon probes for said first region but differing in their target binding sequences, wherein different probes in the set are differently fluorescently labelled, wherein each probe in the set has a single-stranded loop target binding sequence 25 - 50 nucleotides in length and

arms hybrids 4 - 6 nucleotides in length, and wherein each probe in the set is able to hybridise to more than one of the possible variants with different degrees of complementarity, wherein said probes generate detectable fluorescent signals indicative of their hybridization to said first region, and wherein the signals emitted by each of said probes are separably detectable.

7. The kit of claim 6, the gene further comprising a second region which does not overlap with said variant first region, the kit further comprising a supplemental probe having a target binding sequence specific for said second region and not hybridizable to said first region, wherein said supplemental probe is capable of generating a detectable signal indicative of its hybridization to said second region and wherein the signals emitted by each probe in said set of probes and the supplemental probe are separably detectable.

8. The kit of claim 6 or 7, wherein said gene is a mammalian gene or a microbial gene, e.g. a bacterial gene or a mycobacterial gene.

9. The kit of any one of claims 6 to 8, wherein:

(a) said variant is an oncogene; or
(b) said variant is associated with a metabolic disease; or
(c) said variant is associated with an autoimmune disease.

10. The assay according to any of claims 1 to 5 wherein the probe set comprises a limited set of from 3-10 different probes.

11. The kit according to any of claims 6 to 9 wherein the probe set comprises a limited set of from 3-10 different probes.

Patentansprüche

1. Ein homogener Detektionsassay zum Identifizieren einer Variante eines Gens aus einer Menge von möglichen Varianten einer kurzen Nukleotidsequenz, welche einen, sich innerhalb dieser Varianten unterscheidenden ersten Bereich aufweist, in einer Probe, der Assay umfassend:

(a) Bereitstellen eines Aliquots einer Probe, die im Verdacht steht, diese kurze Nukleotidsequenz zu enthalten;
(b) Bilden eines Reaktionsgemisches, das dieses Aliquot beinhaltet;
(c) Sondieren dieses Reaktionsgemisches mit einem Set an molekularen Signalsonden für diesen ersten Bereich, die sich allerdings in ihren Zielsequenzen unterscheiden, unter Hybridisierungsbedingungen, derart, dass jede Sonde in dem Set in der Lage ist, mit mehr als einer der möglichen Varianten mit unterschiedlichen Graden an Komplementarität zu hybridisieren, wobei verschiedene Sonden in dem Set unterschiedlich fluoreszenzmarkiert sind, und wobei jede Sonde in dem Set eine einzelsträngige Loop-Zielsequenz von 25-50 Nukleotiden Länge hat und Hybride von 4-6 Nukleotiden Länge bildet, wobei diese Sonden in diesem Reaktionsgemisch detektierbare Fluoreszenzsignale erzeugen, die ihre Hybridisierung mit diesem ersten Bereich anzeigen, und wobei das von jeder Sonde emittierte Signal getrennt detektierbar ist;
(d) Messen der Intensität dieser Signale; und
(e) Bestimmen des Verhältnisses von normierten Fluoreszenzintensitäten, welche von allen in dem Reaktionsgemisch vorhandenen Paaren an molekularen Signalsonden emittiert werden als ein Anzeichen für die Anwesenheit oder Abwesenheit dieser Variante in dieser Probe.

2. Assay nach Anspruch 1, wobei dieses Reaktionsgemisch ein Amplifikations-Reaktionsgemisch ist, welches das Set an Sonden enthält, wobei der Assay weiterhin umfasst, dass nach der Bildung dieses Amplifikations-Reaktionsgemisches dieser erste Bereich, sofern er in der Probe vorhanden ist, amplifiziert wird; wobei dieses Amplifikations-Reaktionsgemisch gegebenenfalls ein PCR-Amplifikationsgemisch ist, welches ein Primer-Paar und eine DNA-Polymerase beinhaltet.

3. Assay nach Anspruch 1 oder 2, wobei diese kurze Oligonukleotidsequenz eine Länge von bis zu 50 Nukleotiden aufweist oder eine Gensequenz ist, gegebenenfalls ein Säugetier- oder Mikrobengen, beispielsweise ein bakterielles Gen oder ein mykobakterielles Gen.

4. Assay nach einem der Ansprüche 1 bis 3, wobei diese Sonden an vorausgewählten Stellen auf einer festen Oberfläche immobilisiert sind.

5. Assay nach einem der Ansprüche 1 bis 4, wobei:

(a) diese Variante ein Onkogen ist; oder
(b) diese Variante mit einer Stoffwechselerkrankung assoziiert ist; oder
(c) diese Variante mit einer Autoimmunerkrankung assoziiert ist.

6. Kit aus Reagenzien, die zum Identifizieren einer von mehreren möglichen Varianten eines Gens in einer Probe geeignet sind, wobei das Gen einen, sich innerhalb dieser Varianten unterscheidenden ersten Bereich aufweist, dieser Kit umfassend ein Set an molekularen Signalsonden für diesen ersten Bereich, die sich allerdings in ihren Zielsequenzen unterscheiden, wobei verschiedene Sonden in dem Set unterschiedlich fluoreszenzmarkiert sind, wobei jede Sonde in dem Set eine einzelsträngige Loop-Zielsequenz von 25 bis 50 Nukleotiden Länge hat und Hybride von 4 bis 6 Nukleotiden Länge bildet, und wobei jede Sonde in dem Set in der Lage ist, mit mehr als einer der möglichen Varianten mit unterschiedlichen Graden an Komplementarität zu hybridisieren, wobei diese Sonden detektierbare Fluoreszenzsignale erzeugen, die ihre Hybridisierung mit diesem ersten Bereich anzeigen, und wobei die von jeder dieser Sonden emittierten Signale getrennt detektierbar sind.

7. Kit nach Anspruch 6, wobei das Gen weiterhin einen zweiten Bereich umfasst, der nicht mit diesem, sich unterscheidenden ersten Bereich überlappt, der Kit weiterhin umfassend eine zusätzliche Sonde, welche eine für diesen zweiten Bereich spezifische Zielsequenz hat und mit diesem ersten Bereich nicht hybridisierbar ist, wobei diese zusätzliche Sonde in der Lage ist, ein detektierbares Signal zu erzeugen, das ihre Hybridisierung an diesen zweiten Bereich anzeigt und wobei die von jeder Sonde emittierten Signale in diesem Set an Sonden sowie der zusätzlichen Sonde getrennt nachweisbar sind.

8. Kit nach Anspruch 6 oder 7, wobei dieses Gen ein Säugetiergen oder ein Mikrobengen ist, beispielsweise ein bakterielles Gen oder ein mykobakterielles Gen.

9. Kit nach einem der Ansprüche 6 bis 8, wobei:

(a) diese Variante ein Onkogen ist; oder
(b) diese Variante mit einer Stoffwechselerkrankung assoziiert ist; oder
(c) diese Variante mit einer Autoimmunerkrankung assoziiert ist.

10. Assay nach einem der Ansprüche 1 bis 5, wobei das Sondenset ein beschränktes Set von 3 bis 10 unterschiedlichen Sonden umfasst.

11. Kit nach einem der Ansprüche 6 bis 9, wobei das Sondenset ein beschränktes Set von 3 bis 10 unterschiedlichen Sonden umfasst.

Revendications

1. Analyse de détection homogène pour identifier, dans un échantillon, un variant d'un gène parmi un certain nombre de variants possibles d'une courte séquence nucléotidique ayant une première région différant parmi lesdits variants, l'analyse comprenant :

(a) la fourniture d'une portion aliquote d'un échantillon suspecté de contenir ladite courte séquence nucléotidique ;
(b) la formation d'un mélange réactionnel qui inclut ladite portion aliquote ;
(c) le sondage dudit mélange réactionnel avec une série de sondes de balisage moléculaire pour ladite première région mais différant dans leurs séquences de liaison de cible, dans des conditions d'hybridation telles que chaque sonde dans la série est capable de s'hybrider à plus d'un des variants possibles avec des degrés variables de complémentarité, où des sondes différentes dans la série sont marquées par fluorescence de manière différente, et où chaque sonde dans la série a une seule séquence de liaison de cible en boucle simple brin d'une longueur de 25-50 nucléotides et des bras hybrides d'une longueur de 4-6 nucléotides, lesdites sondes produisant dans ledit mélange réactionnel des signaux fluorescents détectables indiquant leur hybridation à ladite première région et où le signal émis par chaque sonde est détectable séparément ;
(d) la mesure de l'intensité desdits signaux ; et
(e) la détermination du rapport des intensités de fluorescence normalisées émises par toutes les paires de

sondes de balisage moléculaire présentes dans le mélange réactionnel comme indication de la présence ou de l'absence dudit variant dans ledit échantillon.

2. Analyse selon la revendication 1, où ledit mélange réactionnel est un mélange réactionnel d'amplification contenant la série de sondes, l'analyse comprenant en outre, après la formation dudit mélange réactionnel d'amplification, l'amplification de ladite première région si elle est présente dans l'échantillon ; éventuellement où ledit mélange réactionnel d'amplification est un mélange d'amplification par PCR qui inclut une paire d'amorces et une ADN polymérase.

3. Analyse selon la revendication 1 ou la revendication 2, où ladite courte séquence oligonucléotidique a une longueur pouvant atteindre 50 nucléotides ou est une séquence de gène, éventuellement d'un gène de mammifère ou microbien, par exemple d'un gène bactérien ou d'un gène mycobactérien.

4. Analyse selon l'une quelconque des revendications 1 à 3, où lesdites sondes sont immobilisées à des emplacements présélectionnés sur une surface solide.

5. Analyse selon l'une quelconque des revendications 1 à 4, où

(a) ledit variant est un oncogène ; ou
(b) ledit variant est associé à une maladie métabolique ; ou
(c) ledit variant est associé à une maladie autoimmune.

6. Kit de réactifs approprié pour identifier un variant parmi de multiples variants possibles d'un gène dans un échantillon, le gène ayant une première région différant parmi lesdits variants, ledit kit comprenant une série de sondes de balisage moléculaire pour ladite première région mais différant dans leurs séquences de liaison de cible, où des sondes différentes dans la série sont marqués par fluorescence de manière différente, où chaque sonde dans la série a une séquence de liaison de cible en boucle simple brin d'une longueur de 25-50 nucléotides et des bras hybrides d'une longueur de 4-6 nucléotides et où chaque sonde dans la série est capable de s'hybrider à plus d'un des variants possibles avec des degrés différents de complémentarité, où lesdites sondes produisent des signaux fluorescents détectables indiquant leur hybridation à ladite première région, et où les signaux émis par chacune desdites sondes sont détectables séparément.

7. Kit selon la revendication 6, le gène comprenant en outre une seconde région qui ne chevauche pas ladite première région du variant, le kit comprenant en outre une sonde supplémentaire ayant une séquence de liaison de cible spécifique pour ladite seconde région et non hybridable à ladite première région, où ladite sonde supplémentaire est capable de produire un signal détectable indiquant son hybridation à ladite seconde région et où les signaux émis par chaque sonde dans ladite série de sondes et la seconde supplémentaire sont détectables séparément.

8. Kit selon la revendication 6 ou 7, où ledit gène est un gène de mammifère ou un gène microbien, par exemple un gène bactérien ou un gène mycobactérien.

9. Kit selon l'une quelconque des revendications 6 à 8, où

(a) ledit variant est un oncogène ; ou
(b) ledit variant est associé à une maladie métabolique ; ou
(c) ledit variant est associé à une maladie autoimmune.

10. Analyse selon l'une quelconque des revendications 1 à 5, où la série de sondes comprend une série limitée de 3-10 sondes différentes.

11. Kit selon l'une quelconque des revendications 6 à 9, où la série de sondes comprend une série limitée de 3-10 sondes différentes.

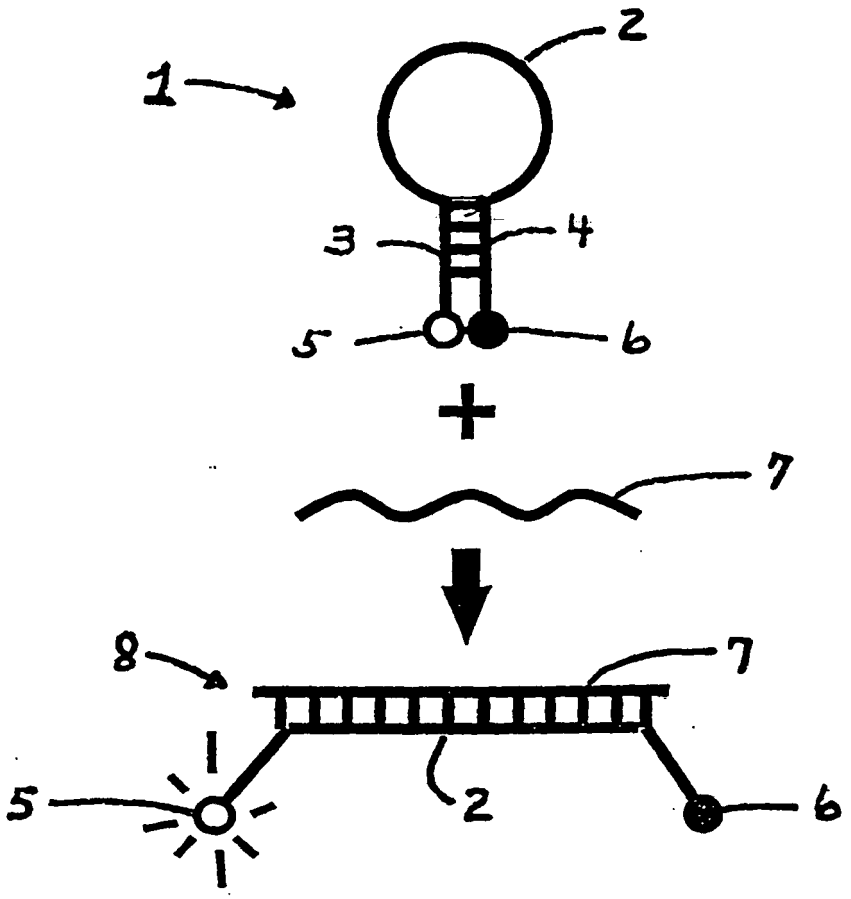

Figure 1

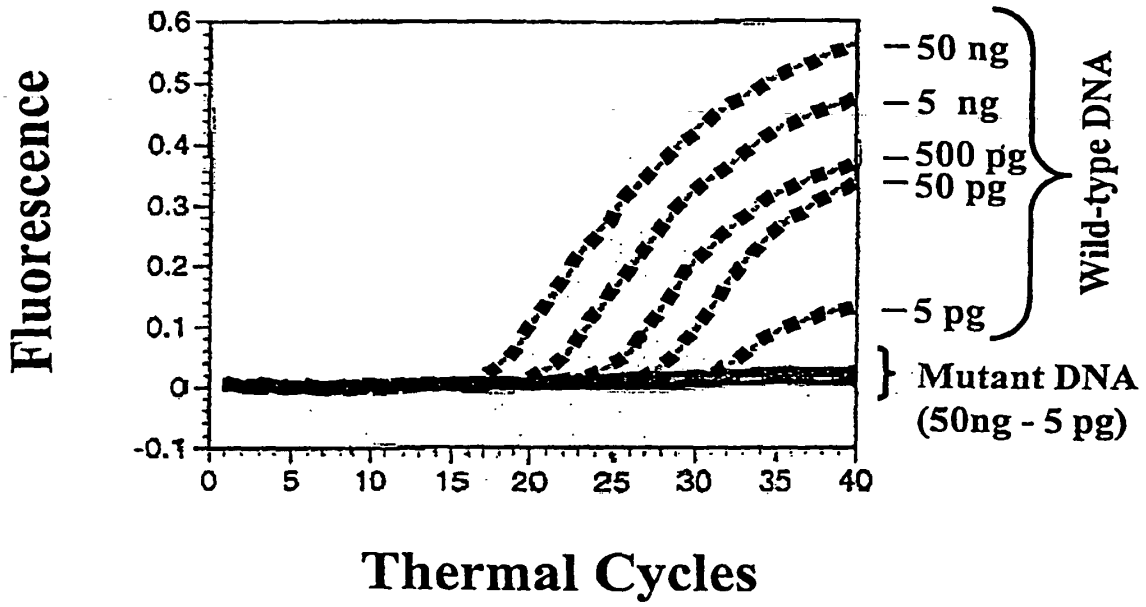
Fluorescence
0.6
0.5
0.4
0.3
0.2
0.1
0
-0.1
0
5
10
15
20
25
30
35
40
Thermal Cycles
50 ng
5 ng
500 pg
50 pg
5 pg
Wild-type DNA
Mutant DNA
(50ng - 5 pg)

Figure 2

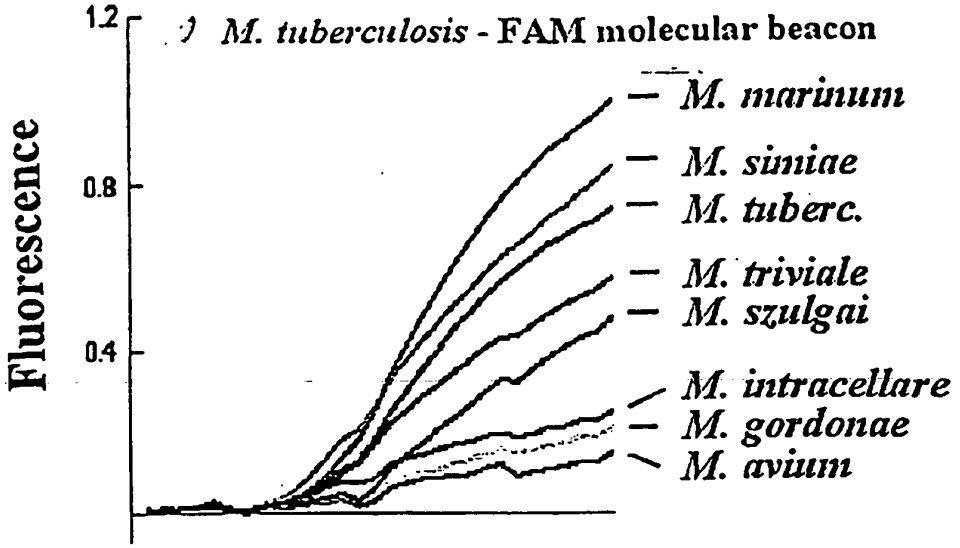

Figure 3A

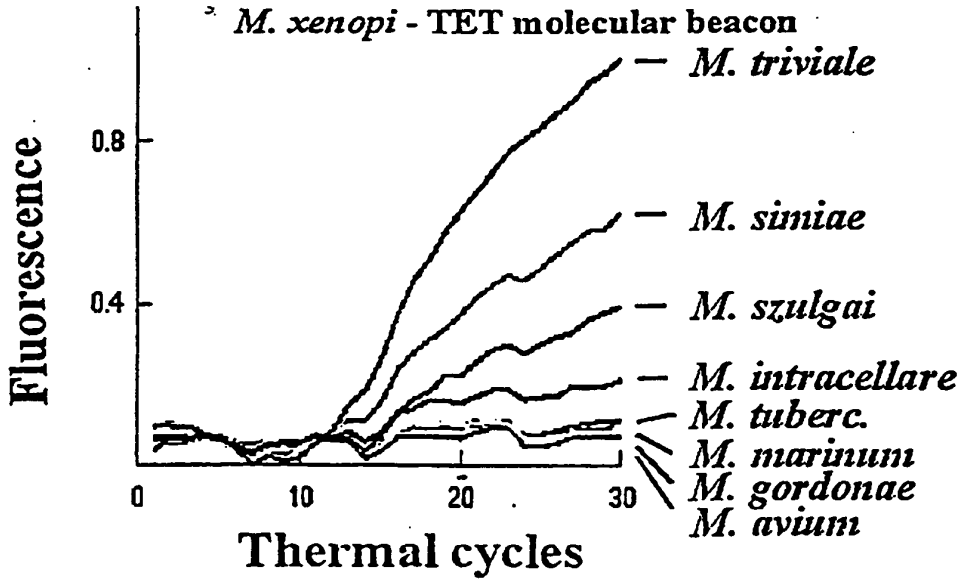

Figure 3B

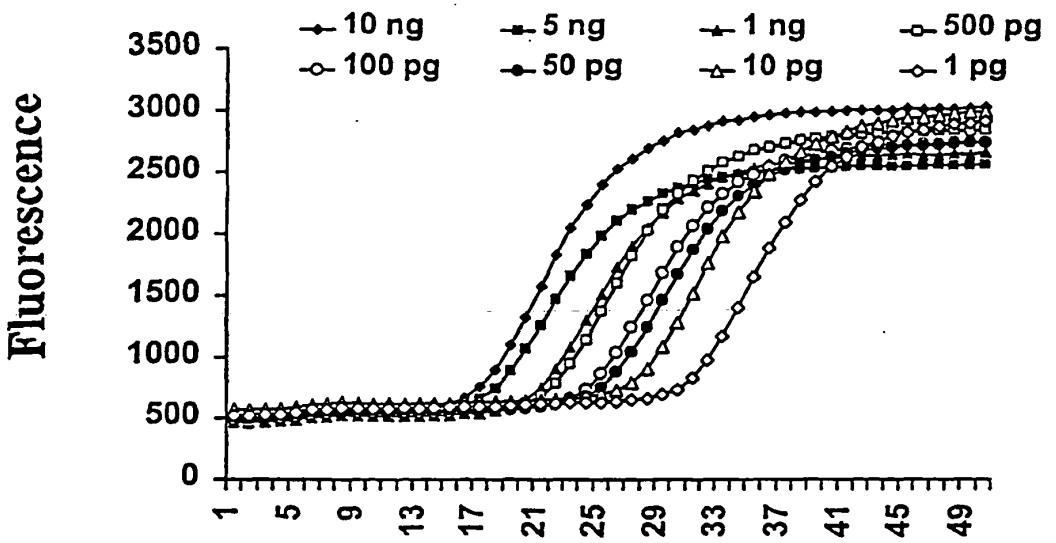

Figure 4A

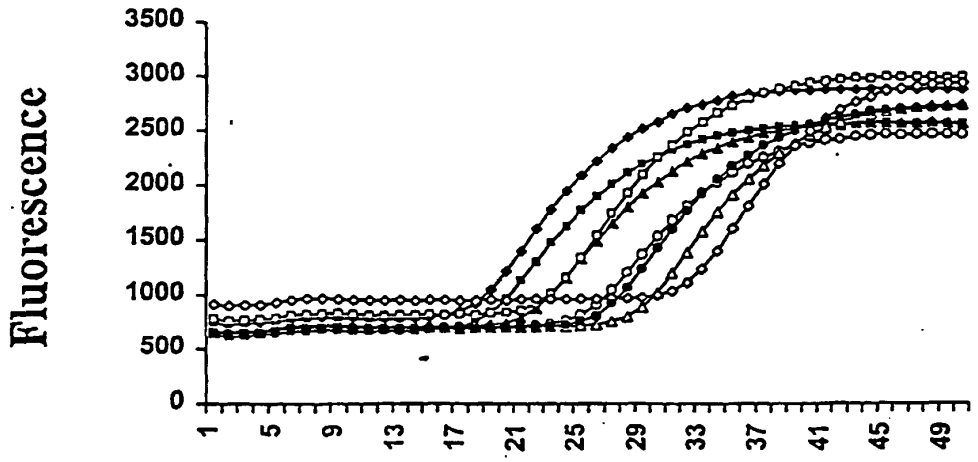

Figure 4B

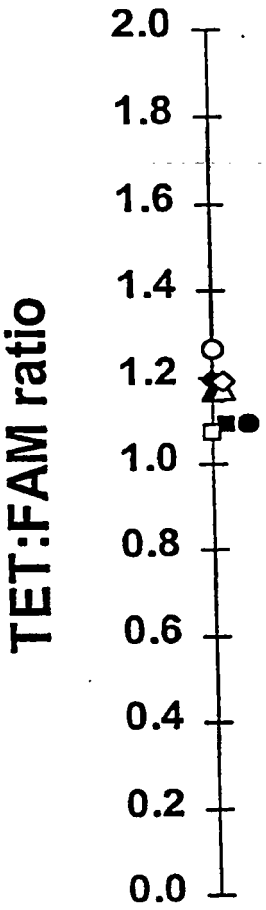

Figure 4C

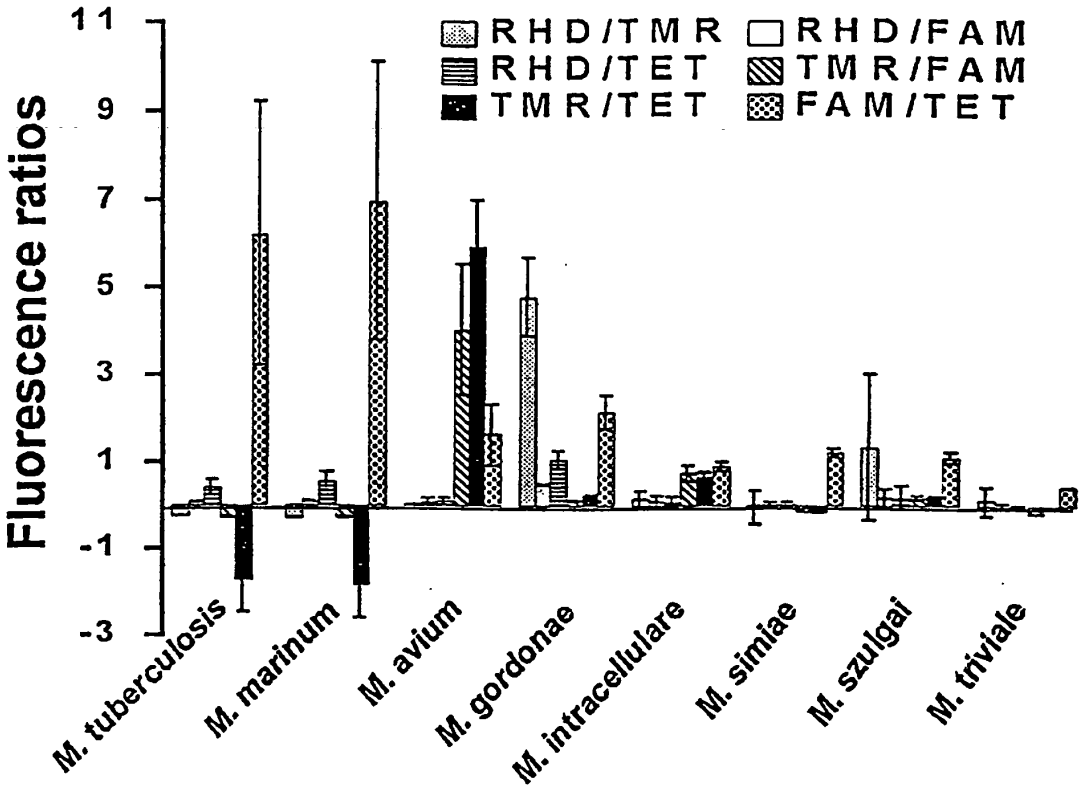
Fluorescence ratios
11
9
7
5
3
1
-1
-3
RHD/TMR
RHD/FAM
RHD/TET
TMR/FAM
TMR/TET
FAM/TET
M. tuberculosis
M. marinum
M. avium
M. gordonae
M. intracellulare
M. simiae
M. szulgai
M. triviale

Figure 5