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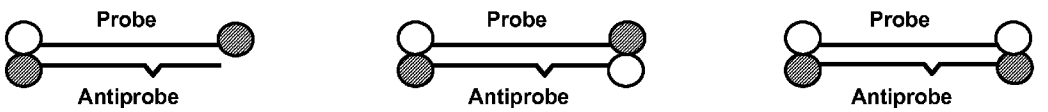
(54) Titre : COMPOSITIONS SONDE: ANTI-SONDE POUR UNE DETECTION D'ADN OU D'ARN A SPECIFICITE ELEVEE

(54) Title: PROBE:ANTIPROBE COMPOSITIONS FOR HIGH SPECIFICITY DNA OR RNA DETECTION



(57) Abrégé/Abstract:
Compositions and methods are provided for amplifying and or detecting nucleic acid targets using labeled polynucleotide probes and antiprobes that interact together and with complementary targets. Competitive thermodynamic interactions of these components result in signaling changes that indicate target frequency, and can provide error-checking functions that facilitate single base discrimination. These probe:antiprobe compositions enable real-time PCR detection, end-point detection and microarray detection of microbial species, drug resistant mutants, and cancer related variants. Some probe:antiprobe systems function as an internal probe, between two primers, and some function as a primer-probe. Some target sequences are discriminated or quantified by employing two probe:antiprobe systems to detect different aspects of the same template. Systems are also provided that enhance target amplification and detection for specific single base variants. A probe may also be modified by introducing a base mismatch to increase thermodynamic discrimination of a correct versus incorrect target differing by a single base.

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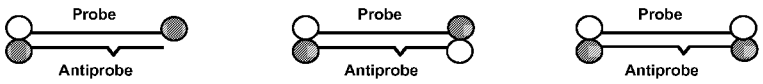

Fig. 5

(57) Abstract: Compositions and methods are provided for amplifying and or detecting nucleic acid targets using labeled polynucleotide probes and antiprobes that interact together and with complementary targets. Competitive thermodynamic interactions of these components result in signaling changes that indicate target frequency, and can provide error-checking functions that facilitate single base discrimination. These probe:antiprobe compositions enable real-time PCR detection, end-point detection and microarray detection of microbial species, drug resistant mutants, and cancer related variants. Some probe:antiprobe systems function as an internal probe, between two primers, and some function as a primer-probe. Some target sequences are discriminated or quantified by employing two probe:antiprobe systems to detect different aspects of the same template. Systems are also provided that enhance target amplification and detection for specific single base variants. A probe may also be modified by introducing a base mismatch to increase thermodynamic discrimination of a correct versus incorrect target differing by a single base.

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PROBE:ANTIPROBE COMPOSITIONS FOR HIGH SPECIFICITY DNA OR RNA DETECTION

CROSS-REFERENCE TO RELATED APPLICATIONS

This application claims priority to U.S. Provisional Patent Application Serial Number 61/534,925 entitled "PROBE:ANTIPROBE COMPOSITIONS FOR HIGH SPECIFICITY DNA OR RNA DETECTION" and filed September 15, 2011.

SEQUENCE LISTING

The present disclosure includes a sequence listing.

FIELD OF THE DISCLOSURE

This disclosure relates to the field of nucleic acid probe technology, and more specifically to compositions and methods to identify and quantify DNA or RNA sequences. In particular it relates to the labeling and detection of gene targets during or post amplification.

BACKGROUND

The detection of targeted polynucleotide sequences is usually based on methods that hybridize labeled DNA probes to a target sequence of interest. To work effectively, the probe-target hybridization products must be washed after hybridization to remove unbound probes and probes that are weakly bound to non-specific targets. However, under the conditions of real-time PCR (US Pat. 4,965,188; US Pat. 5,210,015; US Pat. 5,487,972; US Pat. 5,538,848), a wash step is not feasible, and thus novel probes had to be devised that selectively generate signaling when they are bound to a matching target and that have diminished or quenched signaling when they are unbound and floating free in solution. To achieve this end, there has been reliance on probes that employ the excitation and transfer of fluorescent energy between a donor and an acceptor molecule, such as between two fluorophores, or between a fluorophore and a quencher ([Didenko V, (2001) *Biotechniques* 31:1106-1116, 1118, 1120-1121; Chen *et al.*, (1997) *Proc. Natl. Acad. Sci. USA* 30: 94: 10756-10762). The fluorescence emission spectrum of the donor should overlap the absorption or excitation spectrum of the acceptor. The excited-state energy of the fluorescent donor molecule is then transferred to the acceptor molecule when they are in close proximity (10 to 100 angstroms). However, if the acceptor molecule is itself fluorescent, it provides an emitted signal at a longer wavelength. If the acceptor molecule is an effective quencher, fluorescent signaling is significantly diminished and may be essentially turned off.

TAQMAN.RTM and molecular beacon probes are common probes of this type for real-time PCR detection. In both cases, they serve as an internal probe that is used in conjunction with a pair of opposing primers that flank the target region of interest. The primers amplify the target segment and the probe selectively binds to an identifying sequence between the primer sites, thereby causing increases in fluorescent signaling relative to increases in target frequency. While these probe systems are similar in effect, they employ different detection mechanisms.

A TAQMAN.RTM probe comprises a synthetic oligonucleotide of about 20 to 30 bases that complements a target sequence, and which is labeled on opposing ends with a fluorescent donor and an acceptor (US Pat. 5,538,848). Typically, the 5' end will have a shorter wavelength fluorophore such as fluorescein and the 3' end is labeled with a longer wavelength emitting fluorophore (e.g. TAMRA.RTM) or a non-fluorescent quencher such as BLACK HOLE QUENCHER.RTM. Internal quenchers have also been used. While the TAQMAN:RTM patent has expired, this technology still remains the dominant probe system for real time PCR.

Molecular beacon probes also use fluorescent interactions to detect and quantify a PCR product, with each probe typically having a 5' fluorescent-labeled end and a 3' quencher-labeled end (US Pat. 5,925,517; Tyagi *et al.*, (1996) *Nat. Biotechnology* 14: 303-308). However, molecular beacon probes further include short end segments of about 5 to 7 bases that are complementary and will bind together in solution, forming a stem-loop structure wherein the quencher and fluorophore-labeled ends are brought together and signaling is suppressed.

SCORPION.RTM probes also provide a stem-loop detection mechanism similar to molecular beacons, except that the probe also has a segment attached that serves as an amplification primer (Whitcombe *et al.*, (1999) *Nat. Biotechnol.* 17: 804-807; US Pat. 6,326,145). These probes maintain a stem-loop configuration in the unhybridized state with the fluorophore quenched. When denaturation occurs again followed by annealing, the probe segment binds to the template, thereby opening the stem-loop structure and releasing fluorescence.

Similar to SCORPION.RTM, SUNRISE.RTM. probes comprise a primer attached to a hairpin probe that is extended during amplification. This separates the internal quencher label from the 5' terminal fluorophore (Nazarenko *et al.*, (1997) *Nucl. Acids Res.* 25: 2516-2521).

Conventional dual-labeled probes require selective design and are costly. Their synthesis is difficult and they require manual post-synthesis addition of at least one label as well as high pressure liquid chromatography purification. TAQMAN.RTM and molecular beacon probes also require two opposing primers that flank the probe. To function effectively during the annealing step, TAQMAN.RTM and molecular beacon probes must be longer and have a Tm

(melting temperature) that is 5 to 10 degrees higher than the primers since the probe must bind firmly to the target before extension. This requirement makes it difficult to design or develop dual-labeled probes that can selectively detect SNPs (single nucleotide polymorphisms) or single base mutations, and consequently, false positives are a common problem.

FISH (fluorescent *in situ* hybridization) techniques require four processing steps: 1) the preparation of labeled probes, 2) probe hybridization to fixed denatured targets, 3) the washing of unbound probes, and 4) fluorescent excitation and detection (Barch M.J, editor. "The ACT Cytogenetics Laboratory Manual" 2nd ed. New York: Raven Press; 1991). Careful washing steps are critical to effective detection since the signal to noise ratio is highly dependent on the stringency of washing. Excessive washing can greatly reduce signaling.

Microarray detection resembles FISH detection. Arrays are typically based on printing glass or silicon substrates with bound oligonucleotide cDNA probes; applying fluorescent-labeled DNA or RNA targets which must be hybridized to the probes; washing the arrays stringently; and then detecting the bound targets, usually by laser scanning (Schena *et al.*, (1995) *Science* 270: 467-470; Heller *et al.*, (1997) *Proc. Natl. Acad. Sci. U.S.A.* 94: 2150-2155). Like FISH probes, the wash steps are again complex and time consuming. However, the preparation and labeling of the targets are costly since each target sample is unique, limiting its usefulness for routine microarray-based assays, especially for clinical diagnostics.

SUMMARY

The present disclosure encompasses probe systems suitable for real-time and end-point detection of DNA or RNA sequences with particular emphasis on probes that can discriminate single base variants such as SNPs and point mutations. The present disclosure is especially directed at providing probes suitable for qPCR (real-time PCR) in which small gene segments are exponentially amplified and quantitatively detected. The present disclosure provides a series of probe systems that are structurally and functionally related but which diverge in respective degrees of specificity and or sensitivity, and which can be combined to provide new diagnostic or quantitative capabilities.

A primary aspect of the disclosure is a probe:antiprobe system comprising two labeled oligonucleotides, a probe and an antiprobe, that can interact together. The probe sequence is complementary to the intended target sequence, and the antiprobe sequence is complementary to the probe except for comprising at least one mismatched base in a non-terminal position. The antiprobe is designed to provide an error checking mechanism for the probe. In some embodiments, the probe is generally labeled with a fluorescent emitter and the antiprobe is generally labeled with a fluorescent modulator, such as a quencher, although such labeling can

be reversed and other components, such as a second fluorophore, can serve as a fluorescent modulator. In such embodiments, when probe and antiprobe are bound together, the interacting label moieties are proximate and signaling is diminished, but when the probe binds to a complementary target, fluorophore signaling is released.

This probe:antiprobe system can be configured to enable discrimination of two target sequences that differ by only one base. Accordingly, the probe and antiprobe sequences are engineered to achieve three separate hybridization affinity levels in solution: (i) a first high affinity level between the probe and the intended target, (ii) a second intermediate affinity level between the probe and the antiprobe which is determined by the type and position of the mismatch placed in the antiprobe, and (iii) a third lower affinity level between the probe and an incorrect target that differs by at least one base. The expected hybridization affinity levels are assessed by calculating the Tm and the ΔG of the duplexes expected. The length, sequence and mismatch placement for the components are designed and configured so that the hybridization affinity of the probe:intended target duplex is higher than the affinity of the probe:antiprobe duplex by about 4 or more degrees in Tm and about 2 or more kcal/mol in ΔG, and so that the affinity of the probe:antiprobe duplex is higher than the affinity of the probe:incorrect target duplex by about another 4 or more degrees in Tm and about another 2 or more kcal/mol in ΔG. However, in cases where the inherent thermodynamic difference between the probe:intended target duplex and the probe:incorrect target duplex is limited, the probe may also be modified with an intentional mismatch, advantageously placed about two bases away from the single base variant expected. This probe modification diminishes the hybridization affinity between the probe and the incorrect target due to the proximity of the probe mismatch to the sequence mismatch, the SNP or single base mutant of interest, in the incorrect target. With these various thermodynamic designs, the probe:antiprobe system can achieve discrimination of single base variants and can maintain such discrimination over a range of hybridization conditions and annealing temperatures - particularly when employed for real-time PCR.

The probe:antiprobe system further comprises alternate compositions where the probe is designed to serve as a primer-probe and it replaces one primer, or where the probe serves as an internal probe in conjunction with two flanking primers. The disclosure further comprises a modified probe:antiprobe composition in which the antiprobe is structurally joined to one primer, and this change creates linear versus sigmoid amplification curves by real-time PCR.

Other embodiments of the disclosure provide modified probe:antiprobe compositions for detecting gene segments amplified by an isothermal method, including means for amplification on an array substrate. The disclosure further describes methods for combining two or more

probes together, to detect different target sites simultaneously, or in order to separately detect two aspects of the same target. Finally, the disclosure comprises a probe:antiprobe system combined with a unlabeled blocking probe to enhance the amplification and detection of rare sequence variants such as mutant cancer cells embedded in normal tissue.

BRIEF DESCRIPTION OF THE DRAWINGS

Fig. 1A schematically illustrates the general DDS probe structure and mechanism.

Fig. 1B schematically illustrates the internal DDS (iDDS) system according to the disclosure.

Fig. 1C schematically illustrates the terminal ZIPR DDS system.

Fig. 2A schematically illustrates the FLIP DDS system.

Fig. 2B schematically illustrates the ZIPR DDS:iDDS two probe system to quantify total amplicons and the proportion thereof of a particular variant.

Fig. 2C schematically illustrates the G-Force DDS:iDDS two probe system to quantify total amplicons and the proportion thereof of a particular variant.

Fig. 3A schematically illustrates the Two step "Wild Terminator" ("WTx") system of the disclosure.

Fig. 3B schematically illustrates the One step "Wild Terminator" ("WTx") system of the disclosure.

Fig. 4A schematically illustrates terminal DDS probes combined with ISAM isothermal amplification.

Fig. 4B schematically illustrates internal DDS probes combined with ISAM isothermal amplification.

Fig. 4C schematically illustrates DDS probes with on-chip ISAM amplification.

Fig. 5 illustrates alternate labeling configurations to improve signaling with DDS probe:antiprobe systems: (left) where the probe has both a fluorescence emitter and a fluorescence modulator and the antiprobe has a fluorescence modulator, (middle) where the probe has a fluorescence emitter and a fluorescence modulator and the antiprobe has a fluorescence emitter and a fluorescence modulator, and (right) where the probe has two fluorescence emitters and the antiprobe has two fluorescence modulators.

Fig. 6 illustrates an iDDS probe:antiprobe system according to the disclosure for the detection of the wild-type SNP variant of VKORC1 (Vitamin K epoxide reductase gene). As qPCR descends each cycle from denaturing at 95°C to annealing/detection at about 52-62° C, the probe binds first to the matching target (top) due to correct matching at the SNP site (**bold G**) and thus higher thermodynamic affinity (measured by ΔG and Tm). If no matching target is

available, the probe will then bind secondly to the antiprobe at a lower ΔG and Tm (middle) due to the mismatch engineered in the antiprobe to bring the Tm down about 5-6 degrees and the ΔG down about 2-2.5 kcal/mol. In this case and in most cases, the thermodynamic affinity between the probe and the second target with a non-matching SNP (**bold A**) is significantly lower than the affinity of the probe to the antiprobe, about 5-6 degrees lower in Tm and 2-2.5 kcal/mol lower in ΔG (bottom). Thus, the probe:antiprobe system selectively detects the correct target and inhibits or prevents binding and detection of an incorrect target.

Fig. 7A illustrates the region of exon 21 of the EGFR gene comprising base pair positions 2535-2616 that were amplified to detect the L858R SNP site cancer marker. The mutant template (top) and the wild template (bottom) are shown with the variable 858 SNP site in **bold** and the common primer sequences in **bold**.

Fig. 7B illustrates the 858R probe:antiprobe components to detect the mutant variant of EGFR by qPCR wherein both the probe and the antiprobe are engineered with a mismatched base. The mismatch inserted in the antiprobe (T-T) brings down the thermal affinity of the probe:antiprobe duplex relative to the probe:mutant target duplex. The helper mismatch engineered into the probe (C-C) anticipates a wild target with the 858L SNP variant that is positioned two bases away from the helper mismatch. When the mutant probe encounters such a target, a 3 base "hybridization bubble" occurs as shown (CCG), dropping thermal affinity and preventing false target detection. With effective design, these thermodynamic interactions result in three distinct hybridization levels that differ from one another by about 5-6 degrees in Tm and about 2-2.5 kcal/mol in ΔG. Consequently, as the temperature descends during the annealing step of qPCR, the mutant probe preferentially hybridizes to the mutant target sequence if present due to the higher Tm and ΔG for that duplex as shown (top). The Tm and ΔG for the probe:antiprobe duplex is significantly lower (middle), but still much higher than the Tm and ΔG of the mutant probe:wild target duplex (bottom) due to the SNP mismatch (G-A) and the helper mismatch (C-C) working together to create a multi-base "hybridization bubble". Calculated Tm and ΔG values are depicted in the figure for each hybridization level and for the differences between these levels, based on the Two-State Melting (Hybridization) Analysis program of the DINAMelt Web Server (run at 58 degrees) [N. R. Markham & M. Zuker. DINAMelt Web Server for Nucleic Acid Melting Prediction. *Nucleic Acids Res.* **33**, W577-W581, 2005] It should be noted that the Tm levels for this system are high relative to other commonly employed Tm analysis sources (eg. Operon and IDT websites).

Fig. 8A is a graph showing the fluorescent signal generated by qPCR by using iDDS with a probe oligonucleotide specific for the wild-type SNP variant of VKORC1 with wild-type (W) and mutant variant (M) target nucleotide sequences.

Fig. 8B is a graph showing the fluorescent signal generated by qPCR by using iDDS with a probe oligonucleotide specific for the mutant SNP variant of VKORC1 with wild-type (W) and mutant variant (M) target nucleotide sequences.

Fig. 9 is a graph showing the fluorescent signal generated by qPCR by using iDDS with a probe oligonucleotide specific for the mutant 858R SNP variant of EGFR with wild-type (W) and mutant variant (M) target nucleotide sequences.

Fig. 10A is a graph showing the fluorescent signal generated by qPCR by using iDDS with a probe oligonucleotide specific for the mutant SNP of E. coli O157:H7 with wild-type (W) and (two concentrations) mutant variant (M) target nucleotide sequences.

Fig. 10B is a graph showing the fluorescent signal generated by qPCR by using TaqManMGB with a probe oligonucleotide specific for the mutant SNP of E. coli O157:H7 with wild-type (W) and mutant variant (M) target nucleotide sequences.

Fig. 11A is a graph showing the fluorescent signal generated by qPCR by using iDDS oligonucleotides specific for a gram positive (P) and gram negative (N) bacteria with a gram positive target nucleotide sequence.

Fig. 11B is a graph showing the fluorescent signal generated by qPCR by using iDDS oligonucleotides specific for a gram positive (P) and gram negative (N) bacteria with a gram negative target nucleotide sequence.

Fig. 12 is a graph showing the fluorescent signal generated by qPCR by using ZIPR DDS with a probe oligonucleotide specific for a H3 influenza virus gene target nucleotide sequence.

Fig. 13A is a graph showing the fluorescent signals generated by qPCR by using FLIP DDS probe oligonucleotides specific for a target region in the 16S gene of *Mycobacterium tuberculosis* that differs from the same target region in *Mycobacterium paratuberculosis* by a single base, using four concentrations of the *M. tuberculosis* target nucleotide sequence and a *M. paratuberculosis* control target nucleotide sequence.

Fig. 13B is a graph showing the fluorescent signals generated by qPCR by using a TaqMan probe specific for a target region in the 16S gene of *Mycobacterium tuberculosis* that differs from the same target region in *Mycobacterium paratuberculosis* by a single base, using four concentrations of the *M. tuberculosis* target nucleotide sequence and a *M. paratuberculosis* control target nucleotide sequence.

Fig. 14 is a graph showing the fluorescent signals generated by qPCR by using two-step target enhancement with a "Wild Terminator" blocking probe and an iDDS probe specific for the 858R variant EGFR with a mixed sample of 0.2% 858R target nucleotide sequence and 99.8% wild-type variant target nucleotide sequence.

Fig. 15 is a graph showing the fluorescent signals generated by qPCR by using a G-Force primer-probe with either *M. tuberculosis* wild (W) or mutant (M) templates that do not differ in their primer sequences.

Fig. 16A is a graph showing the fluorescent signals generated by qPCR by using both a non-discriminatory G-Force probe and an iDDS probe that is specific for the wild sequence and using a *M. tuberculosis* mutant (M) template.

Fig. 16B is a graph showing the fluorescent signals generated by qPCR by using both a non-discriminatory G-Force probe and wild-type-specific iDDS probe with differing amounts of *M. tuberculosis* wild (W) versus mutant (M) template.

Fig. 17 is a graph showing the fluorescent signals generated by qPCR by using one-step target enhancement with a "Wild Terminator" blocking probe and an iDDS probe specific for the 858R mutant variant EGFR with a mixed sample of 2% 858R target nucleotide sequence and 98% wild-type variant target nucleotide sequence.

Fig. 18 is a graph showing the fluorescent signal generated by ZIPR and ISAM.

Fig. 19 is a graph showing the fluorescent signal generated by iDDS and ISAM.

Fig. 20 is a digital image of an array detection of ISAM on chip, Cy3 dots, FAM circles.

Fig. 21 is a graph showing the detection of a EGFR Del-19 mutant with a wild only iDDS probe and a non-specific ZIPR probe wherein the curves show two signals in parallel with a 100% wild template, and a flat iDDS signal with a 100% mutant template.

Fig. 22 is a graph showing the detection of EGFR Del-19 mutants with a wild only iDDS probe and a non-specific ZIPR probe wherein the curves show two signals in parallel with a lower iDDS signal with a 50/50 wild/ mutant template, and a flat iDDS signal with a 100% mutant template.

Fig. 23 is a graph showing the detection of EGFR Del-19 mutants with a wild only iDDS probe and a non-specific ZIPR probe with a 100% mutant template with a flat iDDS signal with a 100% mutant template.

Fig. 24 shows the detection scheme for the EGFR Del-19 mutant assay.

The details of some exemplary embodiments of the methods and systems of the present disclosure are set forth in the description below. Other features, objects, and advantages of the disclosure will be apparent to one of skill in the art upon examination of the following

description, drawings, examples and claims. It is intended that all such additional systems, methods, features, and advantages be included within this description, be within the scope of the present disclosure, and be protected by the accompanying claims.

DETAILED DESCRIPTION OF THE DISCLOSURE

Before the present disclosure is described in greater detail, it is to be understood that this disclosure is not limited to particular embodiments described, and as such may, of course, vary. It is also to be understood that the terminology used herein is for the purpose of describing particular embodiments only, and is not intended to be limiting, since the scope of the present disclosure will be limited only by the appended claims.

Where a range of values is provided, it is understood that each intervening value, to the tenth of the unit of the lower limit unless the context clearly dictates otherwise, between the upper and lower limit of that range and any other stated or intervening value in that stated range, is encompassed within the disclosure. The upper and lower limits of these smaller ranges may independently be included in the smaller ranges and are also encompassed within the disclosure, subject to any specifically excluded limit in the stated range. Where the stated range includes one or both of the limits, ranges excluding either or both of those included limits are also included in the disclosure.

Unless defined otherwise, 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 disclosure belongs. Although any methods and materials similar or equivalent to those described herein can also be used in the practice or testing of the present disclosure, the preferred methods and materials are now described.

As will be apparent to those of skill in the art upon reading this disclosure, each of the individual embodiments described and illustrated herein has discrete components and features which may be readily separated from or combined with the features of any of the other several embodiments without departing from the scope or spirit of the present disclosure. Any recited

method can be carried out in the order of events recited or in any other order that is logically possible.

Embodiments of the present disclosure will employ, unless otherwise indicated, techniques of medicine, organic chemistry, biochemistry, molecular biology, pharmacology, and the like, which are within the skill of the art. Such techniques are explained fully in the literature.

It must be noted that, as used in the specification and the appended claims, the singular forms "a," "an," and "the" include plural referents unless the context clearly dictates otherwise. In the specification and claims, reference will be made to a number of terms that shall be defined to have the following meanings unless a contrary intention is apparent.

As used herein, the following terms have the meanings ascribed to them unless specified otherwise. In this disclosure, "comprises," "comprising," "containing" and "having" and the like can have the meaning ascribed to them in U.S. Patent law and can mean " includes," "including," and the like; "consisting essentially of" or "consists essentially" or the like, when applied to methods and compositions encompassed by the present disclosure refers to compositions like those disclosed herein, but which may contain additional structural groups, composition components or method steps (or analogs or derivatives thereof as discussed above). Such additional structural groups, composition components or method steps, etc., however, do not materially affect the basic and *novel* characteristic(s) of the compositions or methods, compared to those of the corresponding compositions or methods disclosed herein. "Consisting essentially of" or "consists essentially" or the like, when applied to methods and compositions encompassed by the present disclosure have the meaning ascribed in U.S. Patent law and the term is open-ended, allowing for the presence of more than that which is recited so long as basic or *novel* characteristics of that which is recited is not changed by the presence of more than that which is recited, but excludes prior art embodiments.

Prior to describing the various embodiments, the following definitions are provided and should be used unless otherwise indicated.

Definitions

The terms and phrases used herein have their art-recognized meaning which can be found by reference to standard texts and journals known to those skilled in the art. The following definitions are provided to clarify their specific use in the context of this disclosure.

The term "complementary" refers to a sufficient number of matching base pairs in an oligonucleotide sequence to interact specifically (hybridize) with the target nucleic acid sequence to be amplified or detected. In the art, a very high degree of complementarity is needed for hybridization specificity and sensitivity, although it need not be 100%.

The term "denaturation" refers to the unfolding and separation of complementary DNA strands, and can be accomplished by heat or denaturant treatment.

The term "detectable moiety" as used herein refers to a label molecule (isotopic or non-isotopic) that is incorporated indirectly or directly into an oligonucleotide to facilitate detection. Synthesis of labeled oligonucleotides can be accomplished by several methods known to those skilled in the art, and various fluorescent molecules are suitable for probe labeling.

The term "detectably labeled" as used herein refers to an oligonucleotide that is labeled with a fluorophore, or other molecular species that elicits a physical or chemical response that can be observed or detected by eye or by an instrument. As used herein, a "label" or "tag" refers to a molecule that, when appended by, for example, without limitation, covalent bonding or hybridization, to another molecule, for example, also without limitation, a polynucleotide or polynucleotide fragment provides or enhances a means of detecting the other molecule.

The term "DNA" as used herein refers to the polymeric form of deoxyribonucleotides (adenine, guanine, thymine, or cytosine) in a single or double-stranded state and includes linear or circular DNA molecules. In discussing DNA molecules, sequences may be described by the convention of giving only the sequence in the 5' to 3' direction.

The terms "DNA amplification" and "amplification" refers to any process that increases the copies of a specific DNA sequence by enzymatic amplification. A commonly used process is the polymerase chain reaction (PCR). The PCR process of Mullis is described in U.S. Pat. Nos. 4,683,195 and 4,683,202. PCR involves the use of a thermostable DNA polymerase, primers, and heating cycles, which separate the DNA strands and exponentially amplify a gene region of interest. Any type of PCR, such as quantitative PCR, RT-PCR, hot start PCR, LAPCR, multiplex PCR, touchdown PCR, *etc.*, may be used. Advantageously, real-time PCR is used. The extension product of the chain reaction will be a discrete nucleic acid duplex with termini corresponding to the ends of the primers employed.

The terms "enzymatically amplify" or "amplify" as used herein refer to DNA amplification. Currently the most common method is the polymerase chain reaction (PCR). Other amplification methods include LCR (ligase chain reaction), strand displacement amplification (SDA); Qβ replicase amplification (QβRA); self-sustained replication (3SR); and NASBA (nucleic acid sequence-based amplification), which can be performed on both RNA and DNA.

The term "flanking region" as used herein refers to a stretch of nucleotides in a sequence that is 5' or 3' of another region of the nucleotide sequence.

The term "fluorophore" as used herein refers to any reporter group whose presence can be detected by its light emitting properties.

The terms "fluorescence quencher" or "quencher" as used herein refers to molecules that interfere with or absorb the fluorescence emitted by a nearby fluorophore. Exemplary quenchers include, but are not limited to, Dabsyl or a BLACK HOLE QUENCHER.RTM that are non-fluorescent aromatic molecules. A quencher can also be a second fluorescent molecule, for example TAMRA (carboxytetramethylrhodamine), that emits at a different wavelength.

The term "hybridization" as used herein refers to the process of association of two nucleic acid strands to form an anti-parallel duplex stabilized by hydrogen bonding between opposing strands. The terms "hybridizing" and "binding" are used interchangeably and is meant the formation of complementary A-T and C-G base pairs between the nucleotide sequences of two polynucleotide segments. The hybridized strands are called a "duplex."

The terms "hybridizing specifically to" and "specific hybridization" and "selectively hybridize to," as used herein refer to the binding, duplexing, or hybridizing of a nucleic acid molecule preferentially to a particular nucleotide sequence under stringent conditions.

The term "hybridizing affinity" as used herein refers to the property of an oligonucleotide to complement with and hybridize to another nucleotide sequence to form a nucleic acid duplex.

The term "immobilized on a solid support" as used herein refers to an oligonucleotide attached to a substrate at a particular location so that it may be subjected to washing or other physical or chemical manipulation without being dislodged. A number of solid supports and immobilizing methods are known in the art, and may be used in the methods of this disclosure.

The term "locked nucleic acid (LNA)" as used herein refers to a modified nucleotide with an extra bridge connecting the 2' oxygen and 4' carbon. The bridge "locks" the ribose in the 3'-*endo* (North) conformation, which is often found in the A-form duplexes. LNA nucleotides can be incorporated into an oligonucleotide to increase the stability of a nucleic acid duplex.

The term "melting temperature "(T_m)" as used herein refers to a temperature at which hybridized duplexes dehybridize and return to their single-stranded state. Likewise, hybridization will not occur between two strands at temperatures above the melting temperature of the resulting duplex. It is advantageous that the difference in T_m of oligonucleotide-fragment duplexes of this disclosure be from about 1 °C to about 10 °C so as to be readily detectable.

The term "mismatched base position" as used herein refers to where, in a duplex nucleic acid, two opposing nucleotide bases do not pair in a complementary manner. For example, an adenine will correctly form hydrogen bonds with thymidine, whereas cytosine or guanidine will form weaker or no hydrogen bonds with adenine, thereby being a mismatch. Alternatively, in a duplexed nucleic acid, a mismatched base position can be due to the addition or deletion of one

member of a complementary pair of opposing bases, or due to the substitution of one member of a complementary pair of opposing bases with a non-natural base, an abasic site or a spacer.

The term "modulated detectable signal" as used herein refers to a detectable signal emitted by a label moiety that is reduced in intensity or otherwise changed such as, but not limited to, a change in wavelength such that the modulated signal is detectably distinct from a unmodulated signal. A modulated signal can be, for example, a quenched signal where some or all of the energy of the unmodulated signal is absorbed by a second label moiety so that the modulated signal is less intense than the original signal. Alternatively, for example, a first signal from a first label moiety may be a stimulant for a second label moiety to emit a signal of a different wavelength.

The term "multi-base non-hybridized region" as used herein refers to a region of a duplexed nucleic acid comprising at least two, and preferably three or four, opposing bases that are mismatched, thereby forming a "hybridization bubble" of non-duplexed bases.

The term "nucleotide" as used herein refers to a sub-unit of a nucleic acid (whether DNA or RNA or an analogue thereof) which may include, but is not limited to, a phosphate group, a sugar group and a nitrogen containing base, as well as analogs of such sub-units. The terms "nucleotide" and "nucleoside" include those moieties which contain not only the naturally occurring purine and pyrimidine bases, *e.g.*, adenine (A), thymine (T), cytosine (C), guanine (G), or uracil (U), but also to modified or analog bases known to those skilled in the art.

The term "oligonucleotide" as used herein refers to a series of linked nucleotide residues comprising a sufficient number of nucleotide bases to be used in a PCR reaction. A short oligonucleotide sequence may be based on, or designed from, a genomic or cDNA sequence and is used to amplify, confirm, or reveal the presence of an identical, similar or complementary DNA or RNA in a particular cell or tissue. Oligonucleotides may be chemically synthesized and may be used as primers or probes. The terms "oligonucleotide" and "polynucleotide" as used herein may also refer to modified or unmodified RNA or DNA.

The terms "nucleic acid," "nucleic acid sequence," or "oligonucleotide" also encompass a polynucleotide. A "polynucleotide" refers to a linear chain of nucleotides connected by a phosphodiester linkage between the 3'-hydroxyl group of one nucleoside and the 5'-hydroxyl group of a second nucleoside which in turn is linked through its 3'-hydroxyl group to the 5'-hydroxyl group of a third nucleoside and so on to form a polymer comprised of nucleosides linked by a phosphodiester backbone. A "modified polynucleotide" refers to a polynucleotide in which natural nucleotides have been partially replaced with modified nucleotides.

The term "Peptide nucleic acid (PNA)" is a non-naturally occurring polymer similar to DNA or RNA where the backbone is composed of repeating N-(2-aminoethyl)-glycine units linked by peptide bonds. The binding between PNA/DNA strands is stronger than between DNA/DNA strands, and thus the T_m ("melting" temperature) of a 6-base thymine PNA/adenine DNA double helix is 31 °C compared to a similar DNA/DNA duplex that denatures at 10 °C.

The term "Polymerase Chain Reaction" or "PCR" as used herein refers to a thermocyclic, polymerase-mediated, DNA amplification reaction employing template molecules, oligonucleotide primers complementary to the template molecules, a thermostable DNA polymerase, and deoxyribonucleotides, and it involves three repeated processes (denaturation, hybridization, and primer extension) that are performed at distinct temperatures and steps. In many embodiments, the hybridization and extension processes can be performed concurrently. The nucleotide sample to be analyzed may be a PCR amplification product provided from a rapid cycling technique described in U.S. Pat. Nos. 6,569,672; 6,569,627; 6,562,298; 6,556,940; 6,569,672; 6,569,627; 6,562,298; 6,556,940; 6,489,112; 6,482,615; 6,472,156; 6,413,766; 6,387,621; 6,300,124; 6,270,723; 6,245,514; 6,232,079; 6,228,634; 6,218,193; 6,210,882; 6,197,520; 6,174,670; 6,132,996; 6,126,899; 6,124,138; 6,074,868; 6,036,923; 5,985,651; 5,958,763; 5,942,432; 5,935,522; 5,897,842; 5,882,918; 5,840,573; 5,795,784; 5,795,547; 5,785,926; 5,783,439; 5,736,106; 5,720,923; 5,720,406; 5,675,700; 5,616,301; 5,576,218 and 5,455,175. Other methods of amplification include, without limitation, NASBR, SDA, 3SR, TSA and rolling circle replication.

The term "polymerase" as used herein refers to an enzyme that catalyzes the sequential addition of monomeric units to a polymeric chain. In advantageous embodiments of this disclosure, the "polymerase" will work by adding monomeric units whose identity is determined by a complementary template of a specific sequence. DNA polymerases such as DNA pol 1 and Taq polymerase add deoxyribonucleotides to the 3' end of a polynucleotide chain in a template-dependent manner, thereby synthesizing a complementary nucleic acid. Polymerases may extend a primer once or may repetitively amplify two complementary strands using two primers.

The term "primer" as used herein refers to an oligonucleotide complementary to a DNA segment to be amplified or replicated. Typically primers are used in PCR. A primer hybridizes with (or "anneals" to) the template DNA and is used by the polymerase enzyme as the starting point for the replication/amplification process. By "complementary" is meant that the primer sequence can form a stable hydrogen bond complex with the template.

The primers herein are selected to be "substantially" complementary to different strands of a target DNA sequence, but they need not reflect the exact sequence of the template. For example, a non-complementary nucleotide fragment may be attached to the 5' end of the primer, with the remainder of the primer being complementary to the strand. Alternatively, non-complementary bases can be interspersed into a primer, as long as there is sufficient complementarity with the target to hybridize and initiate an extension product.

The term "probe" as used herein refers to oligonucleotides that are nucleic acid sequences of variable length, used in the detection of identical, similar, or complementary nucleic acid sequences by hybridization. An oligonucleotide sequence used as a detection probe may be labeled with a detectable moiety. Various labeling moieties are known in the art, such as radioactive, fluorescent, chemiluminescent or electrochemiluminescent compounds.

The term "probe:antiprobe" as used herein refers to a pair of oligonucleotides having nearly or exactly the same number of base positions and having sequences substantially complementary such that, in the absence of a third nucleotide sequence hybridizing to the probe or the antiprobe, said oligonucleotides can form a duplex. It is within the scope of the disclosure for the probe and antiprobe oligonucleotides to be separate molecules or be linked as a region of a single molecular entity.

The terms "quench" or "quenches" or "quenching" or "quenched" as used herein refer to reducing the signal produced by a molecule. It includes, but is not limited to, reducing the signal produced to zero or to below a detectable limit. Hence, a given molecule can be "quenched" by another molecule and still produce a detectable signal albeit the signal is greatly reduced.

The term "qPCR" refers to a real-time polymerase chain reaction, also called quantitative real time polymerase chain reaction (Q-PCR/qPCR/qrt-PCR) which is used to amplify and simultaneously detect the quantity of a targeted DNA molecule. The quantity can be expressed as either a number of copies or a relative amount normalized to the input DNA. Detection proceeds as the reaction progresses in real time unlike standard PCR, where the product of the reaction is detected at its end point. Two common methods for detection of products in real-time PCR are: (1) non-specific fluorescent dyes that intercalate with any double-stranded DNA, and (2) sequence-specific oligonucleotides that are labeled with a fluorescent reporter and permit detection after hybridization to their complementary DNA target.

The term "selectively detecting" as used herein refers to the ability of oligonucleotide probes of the disclosure to distinguish one nucleotide sequence from another by selectively hybridizing to one sequence under the same or similar hybridizing conditions and, when bound to one sequence and not the other, to provide a detectable signal indicating such binding.

The term "spacer" as used herein refers to any molecular entity such as, but not limited to, a multi-carbon spacer, at least one artificial abasic nucleotide, a peptide, or any other abasic extended moiety that may be attached to the end of an oligonucleotide or that can attach two oligonucleotides together, providing means to block polymerase progress over the spacer.

The term "system" as used herein refers to a combination of at least two oligonucleotides that cooperate to selectively hybridize to a target nucleotide sequence and generate a detectable signal indicating the presence of the target sequence. The system may further include primer oligonucleotides useful for the polymerase amplification of a nucleotide sequence from a template nucleic acid to form an amplified amplicon, said amplicon comprising a nucleotide sequence suspected of being a target sequence.

The terms "target" and "target nucleotide sequence" refers to an oligonucleotide that it is desired to detect. The target analyte for use in the methods disclosed may be an isolated oligonucleotide, an oligonucleotide immobilized on a solid support, or in free solution. For application to the methods of the present disclosure, a "target" may refer to any nucleic acid isolated from a plant, an animal or human subject, a bacterial, viral, or unicellular eukaryotic organism, either from the whole organism, a tissue thereof, or from a cultured cell or cells.

The term "template" as used herein refers to a target polynucleotide strand, for example, without limitation, an unmodified naturally-occurring DNA strand, which a polymerase uses as a means of recognizing which nucleotide it should next incorporate into a growing strand to polymerize the complement of the naturally-occurring strand. Templates may be single or double-stranded. In applications of the present disclosure requiring repeated cycles of polymerization, *e.g.*, the polymerase chain reaction (PCR), the template strand itself may become modified by incorporation of modified nucleotides, yet still serve as a template for a polymerase to synthesize additional polynucleotides.

The term "terminator" probe as used herein refers to an oligonucleotide complementary to a nucleotide sequence located within an amplicon, wherein the "terminator" probe has a modified 5′ end resistant to an exonuclease and a modified 3′ end blocked to a polymerase activity The sequence of the "terminator probe" may be selected or modified such that the hybridization affinity of the "terminator" probe to its complementary nucleic acid sequence is greater than the hybridization affinities of a probe oligonucleotide to a target nucleic acid sequence and of the probe oligonucleotide to a complementary antiprobe oligonucleotide.

The term "thermocyclic reaction" as used herein refers to a multi-step reaction wherein at least two steps are accomplished by changing the temperature of the reaction.

The term "thermostable polymerase" as used herein refers to a DNA or RNA polymerase enzyme that can withstand extremely high temperatures, such as those approaching 100°C. Examples of thermostable polymerases include Taq, Tth, Pfu, Vent, and deep vent.

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 of molecular biology. Although methods and materials similar or equivalent to those described herein can be used in the practice of the present disclosure, suitable methods and materials are described herein.

Abbreviations

DDS, DNA Detection Switch; iDDS, internal DNA Detection Switch; EGFR, epidermal growth factor receptor; qPCR, quantitative PCR (real-time PCR); LNA, locked nucleic acid; PNA, peptide nucleic acid; FISH, Fluorescent In Situ Hybridization; Tm, melting temperature; ISAM, isothermal amplification method; SNP, single nucleotide polymorphism, ZNA, Zip nucleic acid.

Description

The probe systems, compositions and methods of the present disclosure provide sensitive and specific detection of DNA or RNA target sequences, particularly for assessing PCR products that are amplified and detected by real-time PCR (qPCR). Several embodiments of the present disclosure facilitate the discrimination of single base variants (single nucleotide polymorphisms-SNPs) that distinguish bacterial and viral pathogens, cancers and genetic conditions, including drug resistant or drug sensitive variants or mutants that defy detection with ordinary real-time PCR probe systems or with hybridization-based microarray probes. Other embodiments facilitate the detection of two aspects of the same amplicon, such as a primer sequence in common and an internal SNP that may be variant, in order to determine the relative frequency of such variant sequences in a sample.

A particularly useful application of the probe:antiprobe systems according to the present disclosure are particular embodiments that selectively enhance the amplification and detection of a specific target sequence in the presence of a significantly greater proportion of a similar sequence differing from the target by just one mismatched base. For example, a biological sample may be obtained from a human or animal patient having a cancer, where the population of cells in a biopsy sample consists of a much greater percentage of normal cells than cancer cells. It is important, therefore, to provide a detectable signal corresponding to the small number of cancerous cells while avoiding false positive signals from the normal cells. Importantly, in such situations, the cancerous cell may differ from the normal cells by a single nucleotide polymorphism. The systems of the present disclosure, therefore, are advantageous in selectively amplifying and detecting target nucleotide sequences from the minority of cells

having the single base change of interest such as a cancer-associated SNP and not the dominant normal (wild-type) sequence. The systems of the disclosure, for example, can detect at least as low as 0.2% of a target species containing a single base mutant in the midst of about 99.8% of a non-mutant target species.

The present disclosure encompasses embodiments of a probe:antiprobe composition, herein termed a DNA Detection Switch (DDS) probe, comprising two labeled oligonucleotides, a probe oligonucleotide and an antiprobe oligonucleotide, that are complementary in sequence and identical in length and that, in the absence of a target nucleotide sequence complementary to the probe oligonucleotide, interact to form a duplex. The probe oligonucleotide comprises a sequence complementary to a target sequence desired to be detected and further includes a first label moiety attached thereto. The antiprobe oligonucleotide of the systems of the present disclosure comprises a nucleotide sequence complementary to, and typically the same length as, the probe oligonucleotide, and a second label moiety attached thereto. It is contemplated that the nucleotide sequence of the antiprobe will include at least one base mismatched with a base of the probe oligonucleotide. It is further contemplated that while the probe and antiprobe sequences that are complementary are also the same length, it is within the scope of the disclosure that other nucleotide sequences may be attached to either the probe or antiprobe. These attached sequences, while extending the probe or antiprobe in length, do not themselves hybridize and interact with the probe or antiprobe, but preferably are selected to complement, for example, a region of an amplicon other than a target sequence of the probe oligonucleotide.

The systems of the disclosure will exhibit either: (i) a modulated signaling state when the probe oligonucleotide binds to the antiprobe and their interacting labeling components are brought together, or (ii) a detectably distinguishable signaling state when the probe oligonucleotide binds to a target nucleotide sequence and the labeling components are separated.

In the various embodiments of the probe:antiprobe systems of the disclosure, and in particular when the systems are used in real-time PCR analyses, the probe oligonucleotide will have a first labeling moiety at the 5' end thereof, wherein said labeling moiety can be, for example, a fluorescence donor (a fluorophore), and the opposing 3' end is blocked to prevent extension of the oligonucleotide by a 5'-3' polymerase. In these embodiments, the antiprobe can have attached at the 3' end a second labeling moiety that is a fluorescence quencher compound. Accordingly, as shown for example in Figs. 1, 2, 5 - 7, in these embodiments, when the probe oligonucleotide and the antiprobe oligonucleotide are associated to form a duplex nucleic acid, the first labeling moiety, i.e. the fluorophore, and the second labeling moiety, i.e.

the fluorescence quenching compound, are brought into close proximity, whereupon the fluorescence emission from the fluorophore is modulated, thereby reducing or eliminating any detectable fluorescence. In the event that the probe is preferentially bound to a target nucleotide sequence and not to the antiprobe oligonucleotide, the fluorescence quencher compound and the fluorophore are spatially separated, the fluorescence emission is no longer quenched, and is, therefore, detectable, indicating the presence of the target nucleotide sequence.

In the case of a matching target sequence, the probe oligonucleotide binds more firmly to the target sequence rather than to the antiprobe sequence, triggering a detectable signal and, in the case of an incorrectly, mismatched target, the probe will bind more firmly to the antiprobe, thereby inhibiting or preventing probe:mismatched target duplex formation and detection of an mismatched target even when hybridization temperatures are suboptimal. The probe sequence may optionally comprise a helper mismatched base that is positioned about 2 bases away from a targeted variant base position desired to be detected, such that if the variant base position does not match the corresponding base in the probe oligonucleotide, a "hybridization bubble" is formed, as shown in Fig. 7B, to improve single base discrimination, and any unlabeled 3' end is optionally blocked to prevent polymerase extension.

In other embodiments of this system of the disclosure, the first labeling moiety of the probe oligonucleotide can be the fluorescence quenching compound, and the second labeling moiety of the antiprobe oligonucleotide can be the fluorophore. It is further contemplated that the first labeling moiety can be a first fluorophore that upon stimulation emits a fluorescence having a first wavelength. When the probe and antiprobe oligonucleotides are in close proximity due to their hybridization to each other, the emitted fluorescence of the first labeling moiety can serve as a stimulation radiation for the second labeling moiety that is also a fluorophore (i.e. a FRET-based system). The stimulated second labeling moiety then can emit fluorescence at a longer wavelength than that of the first fluorescence. Accordingly, there is a difference between (i) the fluorescence wavelength detected when the probe and antiprobe are in association and (ii) the fluorescence wavelength of the probe label moiety that is detectable only when the probe and antiprobe oligonucleotides are dissociated, as when the probe oligonucleotide complexes with a target nucleotide sequence.

With the appropriate selection of the length and sequence of the probe:antiprobe system intended for a particular target of interest, the system includes an inherent error-checking mechanism that thermodynamically favors one of two binding and detection states. State one, which occurs when a target nucleotide sequence is present that matches the probe

oligonucleotide sequence, is where the probe oligonucleotide preferentially binds to the complementary target sequence rather than to the antiprobe oligonucleotide, thereby triggering positive detection. State two occurs when no target, or a target having at least one base mismatch with the probe oligonucleotide sequence, is present, so that the probe oligonucleotide preferentially binds to the antiprobe oligonucleotide, thereby blocking or preventing detection of a mismatched target, even under suboptimal hybridization or PCR annealing temperatures. Examples of such probe:antiprobe systems according to the disclosure have achieved single base discrimination at qPCR annealing temperatures of between about 52 °C to about 62 °C, as shown, for example in Figs. 6-11.

It was further found that such discrimination was maintained, even when the same assays were run at much lower annealing temperatures (i.e. below 50 °C). The antiprobe binding and blocking mechanism, therefore, provides a unique multi-temperature mechanism to prevent or reduce the likelihood of obtaining false positive results. This capability occurs because the differences in competitive binding between probe, antiprobe and target sequences creates three thermodynamic binding levels: 1) a first high level based on strong complementary binding between the probe and a fully matching target; 2) a second intermediate level based on the weaker binding between probe and antiprobe that results from at least one mismatched base position engineered within the antiprobe; and 3) a third lower level based on the strongly reduced thermodynamic binding that generally occurs between the probe and an non-matching target (Fig. 6). The systems of the disclosure, therefore, comprise a probe that binds first to a fully matched target if such a target is present and that binds secondarily to the antiprobe if no correct target is present. Probe binding to an incorrect target, therefore, is effectively avoided or blocked.

When two potential target nucleotide sequences differ by two or more bases, such differences will cause a large divergence in thermodynamic binding between the probe oligonucleotide and correct (matched) target sequence *versus* the probe oligonucleotide and the incorrect (i.e. mismatched) target sequence. In such cases, almost any mismatch inserted in the antiprobe will result in probe to antiprobe binding that is thermodynamically intermediate between (a) probe to matched target binding and (b) probe to mismatched target binding.

However, when the goal is to discriminate a single base difference between a target and another, similar, sequence, and thermodynamic analysis indicates that the expected variants would not create a significant thermodynamic shift, it may be necessary to further modify the probe:antiprobe system by including a helper mismatch in the probe oligonucleotide sequence, typically within two bases of the single base variant site desired to be detected so that probe

binding to the non-matching target opens a multi-base "hybridization bubble" that accentuates thermodynamic differences (Fig. 7B). This modification of the probe can increase single base discrimination where the non-matching base variant of interest has limited effect on either the Tm or ΔG. Sometimes the non-matching base variant will only drop the Tm about 5 °C or less, and the ΔG might also drop only 2 kcal/mol or less. However, when a helper mismatch is introduced, the Tm can drop at least about 10 °C-15 °C and/or at least about 4 to 5 kcal/mol in ΔG relative to the Tm and ΔG characteristic of binding between the probe and the desired target.

It has been found that single base discrimination can be expected if: (i) the antiprobe mismatch with the probe is placed where it will reduce the T_m by at least about 5 °C and/or the ΔG by at least about 2 kcal/mol compared to probe binding to a correctly matching target, and (ii) the non-matching single base mutant or variant further reduces probe to mismatched-target binding by at least about 5 °C more in Tm, and or at least about 2 kcal/mol more in ΔG relative to probe to antiprobe binding. These thermodynamic parameters are generally achieved by selecting a mismatch site lying between the second base from each end and the central 2 or 3 bases of the probe oligonucleotide, and by inserting a mismatched base in a position that otherwise comprises an A, T or C, wherein an T base is typically inserted in an A site, an A base is typically inserted in a T site, or a T base is inserted in a C site (making a weak G-T mismatch). When the system according to the disclosure is used as an internal probe between flanking primers during an amplification procedure such as real-time PCR, any unmodified 3' ends of probe or antiprobe should also be blocked to prevent polymerase extension.

The present disclosure further encompasses embodiments of methods for making probe:antiprobe system compositions for single base discrimination, the methods comprising the following steps: (a) obtaining (i) a probe oligonucleotide complementary to an intended target nucleotide sequence, and (ii) an antiprobe oligonucleotide that has the same number of nucleotide positions as the probe oligonucleotide and a sequence that is complementary to the probe except for at least one mismatched or deficient base position, and where the probe and the antiprobe each have a labeling moiety attached thereto, the labeling moieties being selected as cooperating when the probe is bound to the antiprobe to provide a modulated, i.e. negative or reduced signaling state, and when the probe is bound to a target nucleotide sequence provides a signaling state indicating probe:target duplex formation; (b) determining by thermodynamic analysis the hybridization binding forces between the probe, the antiprobe, and the target sequences; (c) measuring the binding forces between the probe sequence and the

desired target sequence, the probe sequence and the antiprobe sequence, and the probe sequence and a mismatched target sequence, wherein binding forces are defined as ΔG and/or Tm; (d) determining if the probe to antiprobe binding forces are lower than the probe to matching target binding forces, wherein a difference of at least about 2 kcal/mol in ΔG levels and/or at least about 5 °C in Tm levels indicates that the probe will preferentially bind to the matching target sequence and not to the antiprobe; (e) determining if the probe to the non-matching target binding forces are lower than the probe to antiprobe binding forces, a difference of at least about 2 kcal/mol in ΔG levels and/or at least about 5 °C in Tm levels indicating that the probe will preferentially bind to the antiprobe and not to the non-matching target; (f) determining if the differences in binding forces between probe and the matching target *versus* the probe and the antiprobe *versus* the probe and the non-matching target comprise descending levels of thermodynamic binding whereby the probe will bind to a correct target, if available, and secondarily to the antiprobe, whereupon probe binding to an incorrect non-matching target would be avoided or prevented; (g) optionally modifying the antiprobe at one or more base positions to decrease or increase the binding forces between the probe and the antiprobe oligonucleotides; (h) optionally modifying the probe at one or more base positions and within about 2 base positions of a base position corresponding to a single base polymorphism of the target sequence thereby providing a "hybridization bubble" when the probe hybridizes to a region of the target sequence having an SNP variant and thereby decreasing the binding forces between the probe oligonucleotide and the mismatching target; (i) assessing the probe:antiprobe composition by testing with a target sequence having an SNP within the region complementary to the probe oligonucleotide and with a target not having the SNP; and (j) repeating steps (c)-(i), thereby obtaining a probe:antiprobe system identifying a target nucleotide sequence from a similar sequence having at least one nucleotide difference.

Embodiments of the probe:antiprobe systems of the disclosure may include probe or antiprobe oligonucleotides modified by having with one or more components that can increase the specificity of the probe with its corresponding target sequence by increasing complementary binding, such as, but not limited to nucleotides other than adenosine, cytosine, guanine, and thymidine, various non-natural nucleotides, including but not limited to, LNA (locked nucleic acid) or PNA (peptide nucleic acid) or BNA (bridged nucleic acid), and or the structural modifications MGB (minor groove binder), ZNA (Zip nucleic acid) and the like.

This modification of the probe can increase single base discrimination where the non-matching base variant of interest has limited effect on either the Tm or ΔG. Sometimes the non-matching base variant will only drop the Tm about 5 °C or less, and the ΔG might also drop only

2 kcal/mol or less. However, with the introduced mismatched base and the "hybridization bubble" the Tm can drop about 10 °C-15 °C and/or 4 to 5 kcal/mol in ΔG relative to the Tm and ΔG characteristic of binding between the probe and the desired target.

The probe:antiprobe systems of the disclosure may further include a mismatched base position in the probe or antiprobe selected from a natural non-complementary base, a universal base, an artificial base, an extra non-matching base, a missing base, an abasic site, a spacer, a linker or any structural means that can diminish the complementary binding between the probe oligonucleotide and the antiprobe oligonucleotide, or between the probe oligonucleotide and the desired target sequence.

To enhance signaling or quenching, embodiments of the probe:antiprobe systems may further include probe oligonucleotides and or antiprobe oligonucleotides that are labeled on both ends, wherein a probe can comprise a fluorescence emitter and a fluorescence modulator, and an antiprobe can comprise a fluorescence modulator and optionally a fluorescence emitter, or alternatively, a probe may comprise two fluorescence emitters and an antiprobe may comprise two fluorescence modulators. If the probe oligonucleotide comprises a 3' fluorescent emitter, that end may be optionally modified with a spacer in between the probe oligonucleotide and the 3' fluorophore.

Embodiments of the present disclosure further encompass systems that incorporate the probe:antiprobe system into real time PCR assays. These embodiments include, but are not necessarily limited to:

1. *iDDS probes:* For qPCR as illustrated in Fig. 1B, wherein the probe oligonucleotide is selected to be complementary to a target sequence lying between two flanking PCR primers; and wherein any unlabeled 3' end of the probe or antiprobe oligonucleotide is blocked to prevent polymerase extension therefrom. iDDS probes are especially suited for detecting a desired target nucleotide sequence in the presence of related sequences that differ from the desired sequence by a single nucleotide polymorphism (SNP).

2. The probe:antiprobe composition may also comprise labeled probe and antiprobe components that are terminally joined to comprise one molecule, wherein the antiprobe component comprises a sequence that is deficient in complementary binding to the probe component, compared to the affinity of the probe for a target nucleotide sequence.

3. *ZIPR probes:* For use in qPCR as illustrated in Fig. 1C, the labeled probe, herein called a "ZIPR probe", comprises a probe oligonucleotide that comprises a primer sequence and that thereby allows amplification and simultaneous detection of a targeted segment at the terminal end of a PCR amplified product rather than at an internal sequence located between the PCR

primer sites, wherein the 3' end of the ZIPR probe oligonucleotide is not blocked to prevent polymerase extension. The paired antiprobe oligonucleotide also serves to diminish false target detection. Two such primer-probes can be used at the opposite ends of a target desired to be amplified, where they both can comprise the same labeling to provide double signaling. Alternatively, they can each be differently labeled to provide two color signaling.

4. *FLIP probes*: For use in high specificity real-time detection or end-point detection of amplified targets, as illustrated in Fig. 2A, the antiprobe component of the probe:antiprobe system can comprise a labeled segment conjugated to the 5' end of a primer oligonucleotide by an abasic connector such as a spacer. This modification of the probe:antiprobe structure alters primer kinetics so that the detected signaling exhibits linear amplification curves *versus* normal sigmoid amplification curves so that detection and quantitative assessment of a sample can be accurately achieved at the end-point as well as by real-time monitoring during amplification, as shown, for example, in Fig. 13A. The probe and antiprobe sequences can also be fully complementary, without a mismatch, except that the probe is made slightly longer than the antiprobe sequence, by one or more bases. The linear amplification curves produced by this probe:antiprobe composition are comparable to the linear curves produced by LATE PCR. In LATE-PCR, however, linear amplification is achieved by providing primers with unequal concentrations with one primer severely limited in amount resulting in asymmetrical amplification (Sanchez *et al.* (2004) 101: 1933-1938; Wangh *et al.* US Pat. NO.: 7,632,642).

5. *G-Force probes:* For use in real-time PCR, as illustrated in Fig. 2C, is a primer-probe oligonucleotide, herein called "G-Force" probe, having three segments: a labeled probe segment, an antiprobe segment that can fold together with the probe segment, and a target-specific primer segment at the 3' end. The probe segment is 5' labeled with a fluorophore and includes a C-rich sequence of about 6 to about 9 bases. The antiprobe segment is G-rich and complementary to the C-rich segment and comprises about 6 to about 10 bases. When these segments fold and hybridize together due to their complementary sequences, the guanines in the antiprobe segment serve as a quencher to absorb the fluorescent emissions of the probe. The probe and antiprobe segments are joined by an abasic connector such as a spacer that facilitates the folding and binding of these segments together and that prevents copying of the probe segment when the primer-probe is incorporated into an amplicon. The abasic connector can be flanked by one or more A or T bases to facilitate folding and probe to antiprobe binding.

This G-Force probe:antiprobe system exhibits two structural and signaling states: (i) a folded structure and signaling state that occurs when the primer-probe is not associated with another hybridized nucleic acid, where the cytidine-rich segment folds over and binds to the

guanine-rich segment, bringing the fluorescence-emitting label next to fluorescence-absorbing guanines, and (ii) a second structure and signaling state when the primer-probe is incorporated into an amplified target, whereupon the signaling unit is unfolded and fluorescent emissions are released. However, after the first amplification cycle, the target template is permanently extended with a sequence complementary to the guanine-rich antiprobe segment, thus facilitating probe binding and signaling in subsequent amplification cycles. Amplified targets are thereby labeled and detected quantitatively with one fluorophore per amplicon. A similar primer-probe with the same or different labeling can be used on the other end of the amplicon to provide double signaling or two color signaling.

In one preferred embodiment of this system, the G-Force primer-probe has a 5'-3' probe:antiprobe structure comprising FAM-CCCCTCCA-spacer$_{18}$-AGGAGGGGG plus the 3' primer. Due to the extra G on the antiprobe segment, when the C-rich probe segment binds to the G-rich antiprobe segment, the fluorophore will be in the vicinity of at least two G bases. Alternatively, the probe can have complementary sequences containing about two or more C's near the 5' end of the probe segment and about two or more G's near the 3' end of the antiprobe segment.

6. *Double DDS probes:* It is contemplated that two DDS probe systems according to the present disclosure can be used simultaneously to detect different parts of the same amplified target, as schematically shown in Figs. 2B and 2C. Such a system can comprise a labeled primer-probe such as, but not limited to, a ZIPR probe, a G-Force probe, a FLIP probe, and the like, to detect and quantify amplified targets, and combined with an internal iDDS probe that is differently labeled, to detect and quantify those amplified targets that have a specific internal sequence such as a SNP variation.

It is further contemplated that the embodiments of the disclosure may incorporate a plurality of probe:antiprobe systems according to the disclosure that can selectively detect a plurality of target sequences, wherein a positive signal with the first probe relative to a negative or weak signal from the second probe confirms the presence of a variant or mutant sequence comprising the first target sequence.

A suitable internal probe for use in this embodiment can comprise an iDDS probe:antiprobe system. A useful, but not limiting, primer-probe can be the G-Force primer-probe, where the primer-probe quantifies the amplified targets, and the internal probe:antiprobe composition quantifies the frequency of a specific internal target sequence. The use of both probes allows the measuring of the relative frequency of the variant sequence of interest. In real-time PCR, the primer-probe can exhibit a high curve and the internal probe will exhibit a

curve with a lower angle proportionate to the mutant or variant frequency, as shown, for example, in Figs. 16A and 16B.

A common issue in molecular diagnostics is the occurrence of diverse, closely related small mutations that all may have a similar effect on the structure and function of a gene product or on gene expression. For example, lung cancer diagnostics and treatment can be determined by the analysis of a few mutational biomarkers in the EGFR gene and or the KRAS gene. However, while some of these important disease-specific mutants can comprise one single base substitution, such as the EGFR Exon 21 mutation L858R 2573T>G, other cancer biomarkers are more variable although they are confined to a small sequence region. Clinically, knowing which specific deletion or which base substitution is not all that important since the clinical outcomes of any one of a series of such mutations are effectively the same. While sequencing can be performed to determine if any of such closely related mutations are present, sequencing is not effective when the mutant frequency is less than about 10 percent. One way to overcome this problem is to employ two probes directed to the same template, one detecting the wild sequence if present and the other being non-specific and detecting any wild or mutant sequence, whereupon the differences in signaling can indicate the presence of a mutant variant without specifying which mutant variant is present. This two probe strategy can be applied with an iDDS probe or a primer probe specific to the wild type, and a non-specific primer-probe detecting any variants in the same target region.

7. *iDDS and Terminator probes:* Another aspect of the disclosure is that the probe:antiprobe system may be suitably adapted for a two probe target enhancement system suitable for real-time PCR or other amplification-detection methods to selectively amplify and detect a first target nucleotide sequence that differs by one or more bases from a closely related second target nucleotide sequence. In general, the first target sequence is a mutant variant of interest and the second target sequence can be the wild type ("normal") sequence. This two probe system includes a first labeled internal probe:antiprobe system such as an iDDS probe, and a second unlabeled internal "Terminator" probe (termed "Wild Terminator" if specific for a wild-type nucleotide sequence variant) that has a PCR polymerase blocking function, as shown in Figs. 3A and 3B.

The first probe includes a first target sequence, or the complement thereof, and has at least one base position that only matches the first target sequence, but not the second target sequence. The terminator probe comprises the second target sequence, or the complement thereof, and includes at least one base position found in the second target sequence but not in the first target sequence. The terminator probe is further modified at the 5' end to inhibit or

prevent 5' nuclease digestion thereof, and modified at the 3' end to inhibit or prevent 3' polymerase extension therefrom.

The length and/or position of the terminator probe can be selected such that the affinity of the Terminator probe and the second target sequence is substantially greater than the affinity of the first probe and the first target sequence. Preferably, the affinity of the first probe and the terminator probe for the second target nucleotide sequence can differ by at least about 6 °C in Tm and/or at least about 4 or more kcal/mol in ΔG. In addition, the terminator probe may optionally be modified to further enhance binding to its matching target sequence by using one or more artificial nucleotides such as an LNA, a chemical modification such as ZNA or MGB, or a combination thereof.

As a consequence of the thermodynamic differences between the two probes, the Terminator probe will bind more strongly to the second target sequence, and it will inhibit or prevent the amplification of the second target sequence. It will also inhibit or prevent the binding of the first probe to the second target sequence. Therefore, this system will selectively amplify and or detect the first target sequence, thereby providing a target enhancement system useful for detecting rare or low frequency mutants or variants such as in cancer diagnostics, drug resistance or prenatal genetic screening.

The terminator probe can be modified at the 5' end by the attachment of a molecule such as biotin, ZNA, MGB, BHQ, and the like, incorporation of 2'-O-Methyl RNA bases, attachment of a stretch of randomly selected non-complementary bases. The Terminator probe may be modified at the 3' end by the attachment of a blocking molecule (e.g. a phosphate, a spacer, an amino group, and the like), or a string of randomly selected non-complementary bases.

Accordingly, the two probe target enhancement system of the disclosure may be used in a method to selectively amplify and detect a first target sequence that differs by one or more bases from a second target sequence, even if the amount of the second target sequence is significantly greater than that of the first target sequence. Embodiments of this method, therefore, can comprise the following steps: (a) obtaining a detection probe:antiprobe system, an unlabeled Terminator probe, and a pair of flanking primers; wherein: the detection probe:antiprobe system comprises a labeled probe:antiprobe system where the labeled probe includes a first target nucleotide sequence or the complement thereof; the Terminator probe characterized as: (i) including the second target nucleotide sequence or the complement thereof, wherein the binding affinity of the Terminator probe for the second target sequence is greater than the binding affinity of the labeled probe for the first target sequence, (ii) having a 5'

end modified to inhibit or prevent 5' nuclease digestion, (iii) having a 3' end modified to inhibit or prevent 3' polymerase extension, and (iv) having a length greater than that of the labeled probe, and optionally includes internal or terminal modifications to enhance binding such as an LNA or BNA, a ZNA, or a MGB; (b) obtaining a biological sample suspected of comprising the first or second target sequence, or a mixture thereof; (c) pre-amplifying the first target sequence while blocking amplification of the second target sequence with the Terminator probe; wherein this step optionally comprises between about 30 to about 75 cycles of PCR with low temperature annealing at about 48 °C to about 57 °C and wherein each denaturing, annealing or extension step is limited to about 5 seconds or less; (d) combining an aliquot, or a dilute aliquot of the pre-amplified sample, with the pair of flanking primers, and the probe:antiprobe system, said aliquot being about 5 percent or less of the amplification reaction, and a dilute aliquot is a dilution of the aliquot of at least 1:100 and typically in the range of about 1:300 to about 1:1000; and (e) amplifying and detecting the presence of the first target sequence by real-time PCR or other means.

The two probe target enhancement system of the disclosure provides real-time PCR analysis of samples suspected of containing a low frequency of a first target sequence (typically, but not limited to, the mutant sequence of interest) mixed with a relatively high frequency of the second target sequence (such as, but not limited to, a wild-type sequence). Since both target templates are amplified by the same primers, the PCR reaction components (primers, enzymes, etc.) will be exhausted for the more abundant template, to the extent that amplification and detection of the first target sequence will be inhibited or prevented. It has been found, for example, that low frequency mutants in the range of about 2 percent to about 0.002 percent of mutant content were effectively detected. In addition, it is contemplated that this method will be useful in screening for low frequency mutants or variants in blood or other tissues remote from the source.

The method may be further modified by providing another probe:antiprobe composition specific to the second target sequence in step (e) above to confirm the inhibition or blocking of the second target sequence. This modified method can produce two amplification curves: a first curve that indicates the enhanced presence of the first target sequence (typically positive and indicating the mutant of interest), and a second curve that indicates the diminished presence of the second target sequence (typically negative indicating the wild or normal sequence of interest).

8. *DDS primer-probes and Terminator probes:* The two probe target enhancement system of the disclosure can be further modified by replacing one PCR primer with a primer-probe

composition that can non-specifically amplify and detect at least part of the sequence region targeted by the Terminator probe. To achieve this end, the primer-probe comprises a primer sequence that precedes all or part of the target region of the Terminator probe. Since the primer-probe can amplify any wild or mutant sequences within that region, including base substitutions, deletions or insertions, and if the wild sequence has been specifically blocked by the Terminator probe, then a positive signal with the primer-probe indicates that a mutant sequence is present in that target region. Therefore, one assay system can detect multiple sequence variants of interest, and multiple sequence-specific assays are not required. With this system, the primer-probe can be the ZIPR probe, the G-Force probe, the Half-Universal probe, the Universal probe, a SUNRISE.RTM probe or any other primer-probe suitable for real-time PCR detection. This system can be used with a two-step or a one-step procedure, as shown in Figs 3A and 3B.

To confirm non-specific mutant detection, a second probe:antiprobe composition can be used that comprises the second target sequence or its complement. With this modification, a negative or diminished signal with the second probe:antiprobe and a positive signal with the primer-probe can confirm the presence of a variant or mutant sequence in the sample.

Any of the probes of the probe:antiprobe systems of the disclosure can be anchored to a substrate, preferably by a covalent linker, using methods well-known in the art, and the antiprobe is applied in solution along with the unlabeled targets. Single base discrimination can be achieved with multiple targets when using one common hybridization temperature, even though each probe may have been optimized for somewhat different hybridization temperatures. This temperature tolerant feature enables easier design and more reliable performance when detecting multiple high specificity targets such as SNPs or single base mutants.

9. *DDS probes and Isothermal Amplification Method (ISAM):* The present disclosure further provides an isothermal amplification method (ISAM) incorporating the probe:antiprobe system disclosed herein to exponentially amplify a DNA or RNA target and to detect the products in real-time or at the end-point, the method comprising the steps: (a) obtaining a sample containing an RNA or DNA target sequence; (b) adding to said sample (i) a pair of primer oligonucleotides that can amplify a target sequence isothermally, wherein either one primer or both primers includes a RNA polymerase promoter sequence, (ii) a primer-probe oligonucleotide that comprises a first primer sequence, and a matching antiprobe or, optionally a two segment primer-probe including a RNA polymerase promoter sequence and the first primer sequence, and an antiprobe complementary to the RNA polymerase promoter sequence, (iii) a modified primer comprising an RNA polymerase promoter sequence and a second primer sequence or,

optionally, a two segment primer-probe comprising an RNA polymerase promoter sequence and a second primer sequence, and an antiprobe complementary to the RNA polymerase promoter sequence, and (iv) a reaction mix comprising a reaction buffer, a RNA polymerase promoter, a reverse transcriptase, and RNase H; (c) amplifying the target sequence under isothermal or near isothermal conditions, said conditions comprising a single temperature in the range of about 35 °C to about 50 °C, optionally in the range of about 40 °C to about 42 °C, or comprising two alternating temperatures, each of said temperatures in the range of about 35 °C to about 50 °C, optionally alternating between about 40 °C and about 45 °C; and (d) determining the fluorescent signaling periodically during amplification or at a defined end-point to determine the presence and frequency of the amplified target sequence.

With the above probe:antiprobe compositions and amplification method, an RNA target sequence can be amplified directly to generate an amplified DNA product that is appended with an RNA polymerase promoter sequence on one or both ends and a fluorescent donor label on one or both ends. A DNA target product can be similarly amplified, modified and labeled. With this method, maximal exponential amplification can occur quickly in about 20 to 30 minutes, a rate that is faster than typical PCR or real-time PCR methods where each step is rate limited by the cycling conditions used. (Fig. 4A, 18) Although ISAM amplification can be difficult to achieve with some sequence conditions, ISAM target amplification is more robust (producing 20 to 50% more product) when both primers are appended with an RNA polymerase promoter sequence.

In another embodiment of this method of the disclosure, the probe:antiprobe components can be directed to an internal sequence between the primer sites. In this embodiment, the probe can comprise the first labeling component of a fluorescent donor-acceptor pair and a sequence internal to the primer sequences. The antiprobe can comprise the second labeling component of the fluorescent donor-acceptor pair, and a sequence that is partially deficient in complementary binding to the probe. (Fig. 4B, 19) The antiprobe can further comprise multiple base positions that are mismatched or lacking in complementary binding to the probe. This structurally modified antiprobe sequence is required for the low temperature isothermal amplification conditions.

In another embodiment of the above probe:antiprobe compositions, coupled with the ISAM isothermal amplification method, the 5' end of a primer or primer-probe can be anchored to an array substrate, optimally by a covalent linker, and the RNA or DNA targets are isothermally amplified while attached to the chip substrate. (Fig. 4C, 20) With this method multiple targets can be detected as they amplify in real-time or at the end-point and no wash step is required to remove unbound labeled probes.

One aspect of the disclosure, therefore, encompasses embodiments of a system for selectively detecting a target nucleotide sequence, said system comprising at least one probe:antiprobe system comprising: (a) a probe oligonucleotide comprising a nucleotide sequence complementary to a first target nucleotide sequence, and a first label moiety attached thereto; and (b) an antiprobe oligonucleotide comprising a nucleotide sequence fully complementary to the probe oligonucleotide except for at least one mismatched base in a non-terminal position, and a second label moiety, where the second label moiety is attached to the antiprobe oligonucleotide or is a region of the antiprobe oligonucleotide, where: (i) the probe oligonucleotide is configured for greater hybridization affinity for the first target nucleotide sequence than for the antiprobe oligonucleotide; and the antiprobe oligonucleotide is configured for greater hybridization affinity for the probe oligonucleotide than for the second target nucleotide sequence of interest; (ii) in the presence of the first target nucleotide sequence, the probe oligonucleotide and the first target nucleotide sequence form a duplex, whereupon the first and the second label moieties do not interact, thereby providing a first detectable signal; (iii) in the absence of the first target nucleotide sequence, the probe oligonucleotide and the antiprobe oligonucleotide form a duplex, thereby allowing the first and the second label moieties to interact to provide a modulated detectable signal, wherein the first and the modulated detectable signals are distinguishable; and (iv) in the presence of a second target nucleotide sequence differing from the first target sequence by at least one base mismatch, the probe oligonucleotide and the antiprobe oligonucleotide preferentially form a duplex, thereby inhibiting the probe oligonucleotide from forming a duplex with the second target nucleotide sequence.

In embodiments of this aspect of the disclosure, the duplex of the probe oligonucleotide and the first target nucleotide sequence, and the duplex of the probe oligonucleotide and the antiprobe oligonucleotide differ by at least about 2 kcal/mol in ΔG and at least about 4°C in T_m; and wherein the duplex of the probe oligonucleotide and the second target nucleotide sequence, and the duplex of the probe oligonucleotide and the first target nucleotide sequence differ by at least about 4 kcal/mol in ΔG and at least about 8°C in T_m.

In embodiments of this aspect of the disclosure, the probe oligonucleotide sequence can be configured with a mismatched base position about two bases away from an expected mismatch between the second target nucleotide sequence and the sequence of the probe; whereby the two mismatched bases generate an internal two or three base non-hybridized region of the probe oligonucleotide:second target nucleotide sequence duplex, the duplex thereby having a ΔG and T_m less than the ΔG and T_m of the probe oligonucleotide:antiprobe oligonucleotide duplex.

In embodiments of this aspect of the disclosure, the probe oligonucleotide can be attached to a solid substrate.

In embodiments of this aspect of the disclosure, one label moiety can be a fluorescence emitter, and the other label moiety can comprise a fluorescence modulator selected from the group consisting of: a quencher compound, a fluorescent compound, a metallic particle, and a guanine-rich conjugate.

In embodiments of this aspect of the disclosure, the probe oligonucleotide can comprise a fluorescence emitter and either a fluorescence modulator or a second fluorescent emitter, and wherein the antiprobe oligonucleotide comprises a fluorescence modulator and optionally comprises a fluorescence emitter or a second fluorescence modulator.

In embodiments of this aspect of the disclosure, at least one of the probe oligonucleotide and the antiprobe oligonucleotide can comprise at least one of: a non-natural nucleotide, a minor groove binder (MGB), and a Zip nucleic acid (ZNA).

In embodiments of this aspect of the disclosure, the system can be an iDDS probe where the 3' end of the probe oligonucleotide, and optionally of the antiprobe oligonucleotide, can be blocked to prevent polymerase extension; and where the system can further comprise a pair of flanking primers configured for amplifying a nucleic acid region comprising the first target nucleotide sequence.

In embodiments of this aspect of the disclosure, the system can be a Flip probe, where the antiprobe oligonucleotide can further comprise, at the 3' end thereof, a first primer oligonucleotide and the system can further comprise a second primer oligonucleotide, where the first and the second primer oligonucleotides can be configured for amplifying a nucleic acid region comprising the first target nucleotide sequence.

In embodiments of this aspect of the disclosure, the antiprobe oligonucleotide and the first primer oligonucleotide can be linked by an abasic spacer region.

In embodiments of this aspect of the disclosure, the system can be a ZIPR probe comprising a primer-probe amplicon detection system, where the probe oligonucleotide comprises a primer sequence and is configured to cooperate with a primer oligonucleotide to amplify a target nucleotide sequence, whereupon a detectable signal is generated when the probe oligonucleotide is incorporated into an amplicon.

In embodiments of this aspect of the disclosure, the system can further comprise a second probe oligonucleotide, wherein said first and second probe oligonucleotides selectively hybridize to different target nucleotide sequences of a nucleic acid template; and where the two

probe oligonucleotides each can have a label moiety attached thereto, and wherein the label moieties provide two different detectable signals or the same detectable signal.

In embodiments of this aspect of the disclosure, the first probe oligonucleotide cancomprise a primer-probe that is configured for cooperating with a primer oligonucleotide to amplify a first amplicon with a first label, thereby providing a signal relative to amplicon frequency, and wherein the second probe oligonucleotide is either a second primer-probe or an internal probe that can comprise a second label and a sequence complementary to a target sequence; wherein the targeted sequence can comprise a variable sequence segment of the first amplicon, or a variable sequence elsewhere in a nucleic acid template; whereupon the difference in signaling between the first primer-probe and the second probe provides an indicator of the frequency of the variant sequence relative to the frequency of the first amplicon.

In embodiments of this aspect of the disclosure, the at least one probe system can be selected from the group consisting of: a ZIPR primer-probe, an internal iDDS probe or an internal Flip probe.

In embodiments of this aspect of the disclosure, the primer-probe system alternatively comprises a G-Force primer-probe comprising: (i) a 5' fluorescent-labeled probe segment comprising a cytosine-rich sequence of about 7 to 9 bases, (ii) an abasic spacer, (iii) a guanine-rich antiprobe sequence complementary to the cytosine-rich sequence region, and (iv) a primer sequence; whereupon, when the labeled primer-probe oligonucleotide is incorporated into the amplicons generated, a detectable signal is enabled.

In embodiments of this aspect of the disclosure, the system further comprises an ISAM isothermal amplification system suitable for amplifying and detecting a RNA or DNA target sequence, wherein the primer-probe oligonucleotide is configured to cooperate with a flanking primer to amplify the target sequence; wherein the flanking primer further comprises a 5' RNA polymerase promoter sequence; and wherein the amplification-detection system further comprises a RNA polymerase promoter enzyme, a reverse transcriptase enzyme and RNaseH enzyme; wherein the primer-probe oligonucleotide optionally comprises a 5' RNA polymerase promoter sequence; and wherein an antiprobe oligonucleotide optionally comprises a sequence complementary to the RNA polymerase promoter sequence; and wherein said primer-probe oligonucleotides are configured to comprise one or both primers; whereupon signaling and RNA transcription is provided from one or both ends of the amplicon; and optionally, a primer is affixed to a solid substrate.

In embodiments of this aspect of the disclosure, the system further comprises an ISAM isothermal amplification system suitable for amplifying and detecting a RNA or DNA target

sequence, wherein the iDDS probe oligonucleotide is complementary to an internal target sequence comprising about 20 to about 25 nucleotides in length; and wherein the antiprobe oligonucleotide comprises about 10 to about 15 nucleotides in length; and wherein one or two of the flanking primers further comprises a 5' RNA polymerase promoter sequence; and an amplification-detection system comprising an RNA polymerase promoter enzyme, a reverse transcriptase enzyme and RNaseH enzyme; and optionally, a primer is affixed to a solid substrate.

In embodiments of this aspect of the disclosure, the system is configured to detect a variable nucleotide base position of exon 21 of the EGFR gene, said system comprising the oligonucleotides SEQ ID NOs.: 7-12, and 39-41.

In embodiments of this aspect of the disclosure, the system is configured to detect a variable multi-base deletion within codons 746 to 753 of exon 19 of the EGFR gene, said system comprising at least one unlabeled primer, and two probe:antiprobe systems with different labeling; wherein the first probe system comprises a primer-probe complementary to a non-specific first sequence; wherein the second probe system is complementary to the wild sequence at the exon 19 deletion site and inhibits or excludes detection of target templates comprising a multi-base deletion within codons 746 to 753 of exon 19 of the EGFR gene; whereupon the presence and frequency of an exon 19 deletion is assessed by comparing the relative signaling of the two probe systems.

In embodiments of this aspect of the disclosure, the non-specific probe:antiprobe-primer set is SEQ ID NOs.: 56, 57 and 53, or SEQ ID NOs.: 79, 80 and 12; and wherein the deletion-19 wild-only probe:antiprobe-primer set is SEQ ID NOs.: 81, 82, and 53, or SEQ ID NOs.: 54, 55 and 53, and wherein each non-specific probe:antiprobe-primer set and the deletion-19 wild-only probe:antiprobe-primer set can optionally include supplemental primer SEQ ID NO.: 78.

In embodiments of this aspect of the disclosure, the antiprobe oligonucleotide is combined with a Taqman or Molecular Beacon probe assay; wherein the antiprobe oligonucleotide comprises a fluorescence modulator and comprises a sequence partially complementary to a Taqman or Molecular Beacon probe.

In embodiments of this aspect of the disclosure, the probe:antiprobe system is configured to detect a nucleotide variant of exon 19, 20 or 21 of the *EGFR* gene, a *VKORC1* gene, a *CYP2C9* gene, a *uidA* gene of E. coli, a gram positive bacterium 16s gene, a gram negative bacterium 16s gene, a mycobacterium inhA gene, a mycobacterium *rpoB* gene, a mycobacterium *16S* gene, a hemagglutinin (HA) gene of influenza virus, a matrix (M) gene of influenza A virus, a non-structural (NS) gene of influenza B virus, and a KRAS gene.

In embodiments of this aspect of the disclosure, the probe:antiprobe system comprises the nucleic acid sequences selected from the group consisting of: SEQ ID NOs.: 1 and 2, SEQ ID NOs.: 3 and 4, SEQ ID NOs.: 7 and 8, SEQ ID NOs.: 9 and 10, SEQ ID NOs.: 13 and 14, SEQ ID NOs.: 17 and 18, SEQ ID NOs.: 19 and 20, SEQ ID NOs.: 23 and 24, SEQ ID NOs.: 36 and 37, SEQ ID NOs.: 54 and 55, SEQ ID NOs.: 56 and 57, SEQ ID NOs.: 64 and 65, SEQ ID NOs.: 66 and 67, SEQ ID NOs.: 70 and 71, SEQ ID NOs.: 72 and 73, SEQ ID NOs.: 79 and 80, SEQ ID NOs.: 81 and 82, SEQ ID NOs.: 85 and 86, SEQ ID NOs.: 88 and 89, and SEQ ID NOs.: 91 and 92.

In embodiments of this aspect of the disclosure, the system further comprises an unlabeled blocking terminator probe for selectively suppressing qPCR amplification and detection of a first target sequence versus a second target sequence; wherein the terminator probe comprises: (i) an oligonucleotide complementary to the first target nucleotide sequence, (ii) a 5' end modified to resist exonuclease digestion, (iii) a 3' end modified to resist polymerase extension, and (iv) wherein the terminator probe is further configured with a T_m and ΔG that exceeds the T_m and ΔG of the primers and or probes of the qPCR assay by at least about 5 kcal/mol in ΔG and by at least about 5°C in T_m.

In embodiments of this aspect of the disclosure, the system, to enhance binding, the terminator probe further comprises at least one of a non-natural nucleotide, a minor groove binder (MGB), and a Zip nucleic acid (ZNA).

Another aspect of the disclosure encompasses embodiments of a system for qPCR target enhancement, wherein the system comprises a first subsystem and a second subsystem, wherein the first subsystem comprises: (i) the unlabeled terminator probe oligonucleotide complementary to the first target nucleotide sequence, and (ii) a primer pair configured to amplify nucleic acid regions comprising a first and the second target nucleotide sequence; and the second subsystem comprises: (i) a dilute aliquot of the amplification product generated by the first pre-amp subsystem wherein the dilute aliquot comprises about 0.05% or less of the pre-amp amplification product; (ii) at least one probe:antiprobe system complementary to at least the second target nucleotide sequence, and (iii) at least one primer configured to amplify nucleic acid regions comprising the first and the second target nucleotide sequence; whereupon, pre-amplification with the first subsystem and re-amplification and detection with the second subsystem provides means to selectively amplify and detect the second target nucleotide sequence when the frequency of the first target nucleotide sequence exceeds the frequency of the second target nucleotide sequence by a ratio of 20:1 or more.

It should be emphasized that the embodiments of the present disclosure, particularly, any "preferred" embodiments, are merely possible examples of the implementations, merely set forth for a clear understanding of the principles of the disclosure. Many variations and modifications may be made to the above-described embodiment(s) of the disclosure without departing substantially from the spirit and principles of the disclosure. All such modifications and variations are intended to be included herein within the scope of this disclosure, and the present disclosure and protected by the following claims.

The following examples are put forth so as to provide those of ordinary skill in the art with a complete disclosure and description of how to perform the methods and use the compositions and compounds disclosed and claimed herein. Efforts have been made to ensure accuracy with respect to numbers (*e.g.*, amounts, temperature, *etc.*), but some errors and deviations should be accounted for. Unless indicated otherwise, parts are parts by weight, temperature is in °C, and pressure is at or near atmospheric. Standard temperature and pressure are defined as 20 °C and 1 atmosphere.

EXAMPLES

Example 1

Cycling conditions: Real-time PCR was conducted with a Mx4000 instrument (Stratagene, Inc) using HOTSTART-IT PROBE.RTM qPCR master mix (USB, Inc.) (2x) supplemented with 1μl of 25mM $MgCl_2$ in a 20μl reaction. To initiate hot start conditions, the tube was heated to 95 °C for 5 mins followed by 40 cycles of two-step PCR (denaturation at 95 °C for 15 sec, annealing/extension at 58 °C for 1 min). The templates used were ULTRAMER.RTM oligonucleotides (Integrated DNA Technologies, Iowa, USA) comprising the targeted EGFR gene segment with or without the T>G transversion.

Example 2

Internal DDS (iDDS) probe:antiprobe compositions to detect VKORC1 SNP variants: An important SNP variant related to warfarin dosing is located in the *VKORC1* gene encoding vitamin K epoxide reductase, subunit 1,at the site -1639 thereof, comprising a G>A change in the mutant *versus* the wild type. To detect these two SNP variants by real-time PCR, the probes, antiprobes and primers below were used at the following final concentrations:

VK-1639G-Probe: 5'-FAM-CGCACCCGGCCAATG-Phos-3' (SEQ ID NO.: 1) at 200 nM;
VK-1639G-Antiprobe: 5'-CATCGGCCGGGTGCG-BHQ1-3' (SEQ ID NO.: 2) at 400 nM
VK-1639A-Probe: 5'-FAM- ATTGGCCAGGTGCG-Phos-3' (SEQ ID NO.: 3) at 200 nM
VK-1639A-Antiprobe: 5'-CGCACCTGGCCTAT-BHQ1-3' (SEQ ID NO.: 4) at 400 nM
VK-Forward primer: 5'-CCTCTGGGAAGTCAAGCAAG-3' (SEQ ID NO.: 5) at 200 nM

VK-Reverse primer: 5'-AAATGCTAGGATTATAGGCGTGA-3' (SEQ ID NO.: 6) at 200 nM

While the probes and antiprobes contain the targeted single base variants for VKORC1, each antiprobe was modified with a mismatched base (wild antiprobe position 4, mutant antiprobe position 12) to reduce the binding of the probe to the antiprobe relative to the binding of the probe to a correctly matching target. The intended mismatch in probe:antiprobe structure was to achieve an affinity between probe and antiprobe that is intermediate between the affinity between the probe and the correct target vs. the probe and an incorrect target.

Fig. 6 shows how the sequence of the wild probe interacts: (1) with the intended wild target sequence, (2) with the selected antiprobe sequence, and (3) with an incorrect target sequence (in this case the mutant sequence) to create three different levels of thermodynamic affinity as measured by Tm and ΔG. These probes, antiprobes and targets are then subjected to real-time PCR cycling and detection wherein the temperature repeatedly descends from denaturing at 95 degrees to an annealing temperature of about 58 °C, and then back to 95 °C (with or without an extension step at about 72 °C).

Fluorescent signaling was assessed at each annealing step. Probe to correct target binding occurs first at about 5 °C above the annealing temperature, and then, probe to antiprobe binding occurs second at about the annealing temperature. Correct target binding turns on signaling while antiprobe binding turns off signaling. Thermodynamically, probe to incorrect target binding can only occur last, or not at all, since it can only occur effectively at about 5 °C lower than the temperature at which probe to antiprobe binding is occurring. Moreover, since a two to one excess of antiprobes is provided, probe to incorrect target binding is effectively blocked. Typically, primer binding is also optimized for the PCR annealing temperature. Because of these structural and thermodynamic features of iDDS probe:antiprobe based assays, there is little or no opportunity for a probe to bind to an incorrect target that differs by a single base from the intended target.

Cycling conditions: Real-time PCR was conducted with a Mx4000 instrument (Stratagene, Inc) using HOTSTART-IT PROBE.RTM qPCR master mix (USB, Inc.) (2x) supplemented with 1μl of 25mM $MgCl_2$ in a 20μl reaction. To initiate hot start conditions, the tube was heated to 95 °C for 5 min. Then followed 40 cycles of two-step PCR (denaturation at 95 °C 15 sec, annealing/extension at 58 °C for 1 min). The templates used were Ultramers synthesized by IDT comprising the targeted gene segment, with or without the mutant base, and the flanking primer sites.

Figs. 8A and 8B show the amplification curves from four tubes that contained 1,000 copies of the wild or mutant template and that used either the wild or mutant probe. The

positive (upward) curves reflect either detection of the mutant template with the mutant probe (Fig. 8B), or detection of the wild template with the wild probe (Fig. 8A). The flat curves reflect the wild probe with the mutant template or the mutant probe with the wild template. Similar wild and mutant iDDS probe:antiprobe sets for two related diagnostic SNPs, CYP2C9*2 and CYP2C9*3, were also fabricated and they performed with similar results.

CY2-W-probe: FAM-CATTGAGGACCGTGTTCAAGA-Phos (SEQ ID NO.: 64) at 200 nM;
CY2-W-antiprobe: TCTTGAACACGGTCCTCTATG-BHQ1 (SEQ ID NO.: 65) at 400 nM
CY2-M-probe: FAM-CTCTTGAACACAGACCTCAATGC-Phos (SEQ ID NO.: 66) at 200 nM
CY2M-antiprobe: GCATTGAGGACTGTGTTCATGAG-BHQ1 (SEQ ID NO.: 67) at 400 nM
CY2Fprimer: AATTTTGGGATGGGGAAGAG (SEQ ID NO.: 68) at 200 nM
CY2R-primer: GTTTTTCTCAACTCCTCCACAAGG (SEQ ID NO.: 69) at 200 nM
CY3W-probe: FAM-GAGAAGGTCAATGAATCTCTGGAC-Phos (SEQ ID NO.: 70) at 200 nM
CY3W-antiprobe: GTCCTGAGATACATTGACCTTCTC-BHQ1 (SEQ ID NO.: 71) at 400 nM
CY3M-probe: FAM-AGAAGGTCAAGGAATCTCTGGAC-Phos (SEQ ID NO.: 72) at 200 nM
CY3M-antiprobe: GTCCTGAGATACCTTGACCTTCT-BHQ1 (SEQ ID NO.: 73) at 400 nM
CY3F-primer: CCACATGCCCTACACAGATG (SEQ ID NO.: 74) at 200 nM
CY3R-primer: CCTTGGGAATGAGATAGTTTCTGAA (SEQ ID NO.: 75) at 200 nM

Example 3

Internal DDS (iDDS) probes for real time PCR detection of a single base variant of the EGFR gene (at Exon 21 L858R) associated with lung cancer diagnosis and therapy: To detect the EGFR Exon 21 mutant codon L858R suspected of being present in a nucleic acid sample, and possibly in the presence of the 858L wild-type (normal) codon sequence, the following oligonucleotide probes, antiprobes and PCR primers were synthesized and used at the indicated final concentrations:

EGFR 858R probe: FAM-CAGATTTTGGCCGGGCCAAACTG-Phos (SEQ ID NO.: 7) at 200 nM
EGFR 858R antiprobe: CAGTTTGGCCCGCCCAATATCTG-BHQ1 (SEQ ID NO.: 8) at 400 nM
EGFR 858L probe: CalRed610-CAGATTTTGGGCTGACCAAACTG-Phos (SEQ ID NO. 9) at 200 nM
EGFR 858L antiprobe: CAGTTTGGCCAGCCCATAATCTG-BHQ2 (SEQ ID NO.: 10) at 400 nM
For. primer: GAAAACACCGCAGCATGTC (SEQ ID NO.: 11) at 200 nM
Rev. primer: CTGCATGGTATTCTTTCTCTTCC (SEQ ID NO.: 12) at 200 nM

While the above probes and antiprobes contain the targeted single base variants at codon 858, each antiprobe also includes an additional mismatched base relative to the corresponding probe sequences at mutant 858R antiprobe position 18 (from the 5' terminus)

and at the wild 858L antiprobe position 18(from the 5' terminus) that reduces the binding affinities of the antiprobes to their matching probes (shown in Figs. 7B).

For these particular targets, the thermodynamic difference between (i) the binding of the probe and the correct target and (ii) the probe and the incorrect target was low. The probes, therefore, were each designed to include an additional helper mismatch located two bases away from the variant base site desired to be detected (at variant 858R probe position 11, and at wild 858L probe position 15, respectively). In the event of hybridization of a probe to an incorrect target, a three-base "hybridization bubble" is formed (CCG), preventing formation of the duplex and favoring hybridization to the correct target sequence or to its corresponding antiprobe oligonucleotide.

Fig. 9 shows amplification curves from two tubes that contain 1,000 copies of the mutant template. The positive (upward) curve reflected detection of the single base mutant with the 858R variant probe. The flat curve reflected using the wild probe, which does not detect the mutant template.

Example 4

Real time PCR using iDDS probes for the detection of pathogenic O157:H7 E. coli based on detecting the uidA +93 and comparison with a TaqMan-MGB probe: The probe, antiprobe, and primers were fabricated with the following sequences and labeling, and were used at the indicated final concentrations:

Mutant O157 *uidA* probe: FAM-CACCAACGCTGCTCAATTC-Phos (SEQ ID NO.: 13) at 200nM
Mutant antiprobe: GAA TTGAGCTGCGTTGGTG-BHQ1 (SEQ ID NO.: 14) at 400nM
uidA-For. primer: CAGTCTGGATCGCGAAAACTG (SEQ ID NO.: 15) at 200nM
uidA-Rev. primer: ACCAGACGTTGCCCACATAATT (SEQ ID NO.: 16) at 200nM
Cycling conditions: Real-time PCR was conducted as described in Example 1 except that qPCR was run for 60 cycles and the annealing/extension step was at 60 °C for 1 min.

Fig. 10A shows raw amplification curves from four tubes using the O157 mutant probe to detect two concentrations (50 copies and 5 copies) of the *E. coli* mutant template (positive curves) *versus* 50 and 5 copies of the *E. coli* negative template (flat curves). No false positive detection is seen with the wild template for 60 cycles.

In comparison, Fig. 10B shows amplification curves from two tubes using a commercially available Taqman-MGB probe specific for the O157 mutant to detect 50 copies of the mutant template (positive(upward) curve) versus 50 copies of the control template (predominantly flat curve). The control template, not having the pathogen-associated polymorphism, yielded a number of false positive results (curve angling up) with the Taqman-MGB probe at 38 cycles.

Example 5

Real time PCR using iDDS probes for the detection of Gram negative (GN) vs. Gram positive (GP) bacteria: The probe, antiprobe, and primers were fabricated with the following sequences and labeling, and were used at the indicated final concentrations:

GP probe: FAM-AAGGGGCTTGATGATTTGACGT-Phos (SEQ ID NO.: 17) at 200nM
GP antiprobe: ACGTCAAATCTTCATGCCCCTT-BHQ1 (SEQ ID NO.: 18) at 400nM
GN probe: CalRed610-AAGGGCCATGATGACTTGA-Phos (SEQ ID NO.: 19) at 200nM
GN antiprobe: TCAAGTCTTCATGGCCCTT-BHQ2 (SEQ ID NO.: 20) at 400nM
For. primer: TCCCGCAACGAGCGCAAC (SEQ ID NO.: 21) at 200nM
Rev. primer: CAGCCATTGTAGCACGTGTGT (SEQ ID NO.: 22) at 200nM

Cycling conditions: Real-time PCR was conducted as described in Example 1 with the annealing/extension step at 58 °C for 1 min. Both probes were used together in the same tube and each tube contained a different template.

Fig. 11A shows two curves in one tube with both the gram positive and the gram negative probe. The positive (upward) curve indicates the gram positive probe detecting gram positive templates from about 1.3×10^4 cells, and the flat curve reflects that the gram negative probe shows no false positive detection with the same template.

Alternatively, Fig. 11B shows two curves in one tube wherein the positive curve reflects the gram negative-specific probe detecting the gram negative template from about 2.6×10^5 cells. The flat curve reflects the gram positive probe showing no false positive detection with those same templates.

In additional experiments, the Gram Negative probe (SEQ ID NO.: 19) was modified with FAM fluorescent labeling at the 5' end and with BHQ1 quencher labeling at the 3' end, and the Gram Negative antiprobe (SEQ ID NO.: 20) was labeled with BHQ1 at the 3' end, and optionally with FAM at the 5' end. Both modifications improved detection by qPCR, producing higher levels of exponential fluorescent signaling and lower background levels.

Example 6

Real time PCR using ZIPR DDS probes for the detection of H3N2 influenza: The ZIPR H3N2 probe was targeted to a site in the hemagglutinin (HA) segment of H3N2 influenza genomes and was FAM labeled. The probe comprised a target-specific primer sequence. The antiprobe is BHQ1-labeled and is largely complementary to the probe. The primer was used in conjunction with the probe and antiprobe to amplify and detect H3N2 samples, and they were used at the following final concentrations:

ZIPR H3 Probe: FAM-CTGGTTCAGAGTTCCTCAACA (SEQ ID NO.: 23) at 200nM

ZIPR H3 antiprobe: TGTTGATGAACTCTGAACCAG-BHQ1 (SEQ ID NO.: 24) at 400nM
H3 primer: CCATCAAGGATCTGATGAGGA (SEQ ID NO.: 25) at 200nM
Cycling conditions: Real-time PCR was conducted as described in Example 1.

Fig. 12 shows an amplification curve from an H3N2 infected patient sample using the ZIPR H3 probe above.

Example 7

Real time PCR using FLIP DDS probes for the detection of 16S of Mycobacterium tuberculosis versus 16S of Mycobacterium paratuberculosis: The following FLIP DDS probe for the specific detection of *Mycobacterium tuberculosis* species was targeted to a site in the 16S gene that differs by one base from the 16S gene of *Mycobacterium paratuberculosis*. The probe was 3'-FAM-labeled and comprised an internal target sequence. The antiprobe component was 5'-BHQ1-labeled and was conjugated to a 3' primer sequence, in this example to the forward primer.

Only one flanking primer was used in conjunction with the FLIP probe components. During target amplification, the probe bound to the target sequence, leaving the antiprobe behind since it was attached to one primer that is incorporated into the amplicon. Thus the probe could flip forward to its target site, triggering fluorescent detection. The assay used a second primer that was not encumbered with an antiprobe. Comparison was made with a Taqman probe for the same target site, using both the forward and reverse primer and the same test samples. The primer and probe components and final concentrations were as follows:
FLIP probe: TAGGACCACGGGATGCATGTCTT-FAM (SEQ ID NO.: 26) at 125nM
FLIP antiprobe-primer: dT-BHQ1-AAGACATGCATCCCGTGGT-spacer9-GGGATAAGCCTGGGAAACTG (SEQ ID NO.: 27) at 200nM
Taqman probe: FAM-CATGTCTTGTGGTGGAAAGC-BHQ1 (SEQ ID NO.: 28) at 100nM
For. primer: GGGATAAGCCTGGGAAACTG (SEQ ID NO.: 29) at 200 nM
Rev. primer: ACCCCACCAACAAGCTGATA (SEQ ID NO.: 30 at 200nM
Cycling conditions: Real-time PCR was conducted as described in Example 1.

Fig. 13A shows amplification curves from five tubes using the FLIP DDS probe specific for 16S *M. tuberculosis*. The four positive (upward) curves reflect detection of four samples of *M. tuberculosis* serially diluted 10:1. The negative curve reflects a sample of *M. paratuberculosis* that differs by one base in the probe region.

Fig. 13B shows curves from five tubes using a Taqman probe specific for 16S *M. tuberculosis* at the same target region as the FLIP probe shown in Fig. 13A. The four high positive curves are from the same four serial dilutions of *M. tuberculosis* as shown above. The

low positive curve overlapping the other curves is from the same *M. paratuberculosis* control sample as in Fig. 13A. This illustrates false positive detection occurring with the Taqman probe. The FLIP probe is more stringent and avoids such false positive detection.

Example 8

Real time PCR using G-Force DDS probe for the detection of the M. tuberculosis rpoB gene: A G-Force DDS probe for tuberculosis was designed to detect a region in the *rpoB* gene that encompasses codons 526 to 533. A generic probe:antiprobe signaling unit was joined to one primer and functioned in conjunction with the other flanking primer to amplify and detect the target site. The G-Force signaling unit comprised a FAM-labeled cytidine-rich probe segment, a spacer flanked by A's, and a guanine-rich antiprobe segment (as shown in SEQ ID NO.: 31).

In the absence of a target sequence, the probe and antiprobe segments fold together, with the spacer in between, due to the binding of complementary sequences. Since this brings the fluorophore next to a string of guanines, fluorescence was significantly diminished. But when the primer/probe unit was incorporated into the product, the antiprobe segment is copied, thereby preventing the probe segment from folding next to the antiprobe segment, and therefore signaling is released.

The G-Force probe and primer sequence and final concentrations are:
GF primer/probe: FAM-CCCCTCCA-spacer18-AGGAGGGGG-CCGCTGTCGGGGTTGAC (SEQ ID NO.: 31) at 100nM; Reverse primer: CACGCTCATGTGACAGACC (SEQ ID NO.: 32) at 200nM

The G-Force probe was used with two templates. One containing a single base mutant at codon 526 (tac), the other the wild sequence. The G-Force probe did not differentiate these two templates.

Cycling conditions: Real-time PCR was conducted as described in Example 1.

Fig. 15 shows the results from two tubes with the probe above showing positive curves with both the wild and mutant template.

Example 9

G-Force DDS probe combined with an iDDS probe for detection of a single base mutant in the rpoB gene of M. tuberculosis: The G-Force DDS probe from Example 8 above was used in conjunction with an iDDS probe targeting the wild sequence for the 526 codon of rpoB. The iDDS probe components and concentrations are below. This assay was intended to detect if a mutant is present at codon 526, the primary site for rifampicin resistant mutants, without having to detect each specific mutant with a different iDDS probe. Thus the iDDS probe gives a flat curve if a 526 mutant is present, while the G-Force probe will still give a positive curve. This

positive/negative result confirms that the 526 itself codon is present, and then shows that it contains a mutant base, regardless of which base variant is present. While this positive/negative result confirms that only a resistant mutant is present, the assay can be ambiguous if the sample contains significant quantities of both wild and mutant templates and two positive curves are detected.

iDDS probe: CalFluorRed610-CGGGGTTGACCCACTAGCG-phos (SEQ ID NO.: 33) at 200nM

Antiprobe: CGCTTGTGGGTCTACCCCG-BHQ2 (SEQ ID NO.: 34) at 400nM

Cycling conditions: Real-time PCR was conducted as described in Example 1.

Fig. 16A shows one tube with the two probes above and with a mutant template for the 526 site. The G-Force probe showed a positive curve detecting the 526-533 region, but the more specific wild iDDS probe for the 526 site did not show a positive curve since the 526 site is mutant. This assay thus provides an index of *rpoB* 526 mutant status without detecting a specific mutant.

A G-Force probe can be combined with a iDDS probe to quantify the frequency of a specific mutant in a sample. The G-Force probe detects all amplicons of the target region while the iDDS probe only detects those amplicons that comprise the mutant sequence. Thus the signal is consistently high for the G-Force probe with either wild or mutant templates, while the signal height of the iDDS probe is in proportion to the frequency of the mutant template versus the wild template, and provides an indication of the relative proportions of wild versus mutant cells in a population of cells. The same would be true in reverse if the iDDS probe detected the wild and not the mutant sequence.

This capacity is shown with a G-Force probe for the *inhA* gene of *M. tuberculosis* combined with an iDDS probe for the wild *inhA* sequence at the site of a common drug resistant mutant using the following probes and primers:

For. primer: GCTCGTGGACATACCGATTT (SEQ ID NO.: 35) at 200nM; inhA iDDS probe: CalRed610-CCGACAACCTATCGTCTCGCC-Phos (SEQ ID NO.: 36) at 200nM; inhA antiprobe: CGAGACGATAGGTTGTCGG-BHQ2 (SEQ ID NO.: 37) at 400nM; InhA G-Force primer-probe: CCCCTCCA-spacer18-AGGAGGGGGTCCGGTAACCAGGACTGAAC (SEQ ID NO.: 38) at 100nM

Fig. 16B shows a test where two tubes were run with the wild and mutant template mixed at either 75% wild:25% mutant or 25% wild:75%mutant. While the curves for the G-Force probe were the same for either template, the curves for the iDDS probe differed in height proportional to the percent wild template present.

Example 10

iDDS probe-based mutant detection enhanced with "Wild Terminator" method: Mutant detection with iDDS probes could be enhanced by a pre-amplification step that selectively amplified the targeted mutant templates and blocked the amplification of wild templates. After the pre-amp step, the reaction product was diluted and a small dilute sample was transferred to a real-time PCR reaction. Because of this process, wild type templates were almost eliminated and the qPCR reaction started with an amplified quantity of mutant templates. The result was that a sample with a low frequency of mutant templates could be efficiently detected by qPCR with an iDDS probe even if the original sample contained an abundance of wild templates that would otherwise obscure mutant detection.

The pre-amplification reaction used a set of primers flanking the targeted region, and the final reaction used a primer set that was partially or fully between the outer primers. The first, pre-amplification procedure was run in a standard PCR machine for 30 to 70 short cycles. One microliter of the first reaction was generally diluted with 100 to 500 microliters of water or buffer, and then a one microliter sample was transferred to a second reaction in a real-time PCR machine.

The first step used a Wild Terminator blocking probe comprising an unlabeled oligonucleotide that was complementary to the wild sequence and about 22 to 28 bases long - about 2 to 5 bases longer than the iDDS probe. The 5' end was modified to prevent 5'-nuclease digestion the attachment of a blocking moiety such as a biotin molecule, a ZNA, a MGB, or an arbitrary string of non-complementary bases (about 5-10). The 3'-end was modified with a molecule that prevents extension of the probe using such as a phosphate, an amino group, or a spacer. The Tm of the blocking probe was typically at least 5 °C higher than the iDDS probe so that any wild templates would bind to it strongly.

The blocking "Wild Terminator" probe, thus acted like an antiprobe in reverse, blocking wild templates while allowing iDDS probes for the mutant sequence to bind to, and detect, mutant templates. This example illustrates enhanced detection of the EGFR Exon 21 mutant site L858R (T>G) using the same iDDS probes described in Example 2 but providing mixed templates with a low frequency (0.2%) of the mutant sequence variant relative to the wild sequence variant.

The first pre-amplification step used the following templates and components: Templates: 10,000 copies wild EGFR Exon 21 858L; 20 copies mutant EGFR Exon 21 858R (0.2%). Outer forward primer: AGCCAGGAACGTACTGGTGA (SEQ ID NO.: 39) at 100nM

Outer reverse primer: TGCCTCCTTCTGCATGGTAT (SEQ ID NO.: 40) at 100nM; Terminator blocking probe: Biotin-CTTTCCCACCAACGCAGATCAATTCCA-phos (SEQ ID NO.: 41) at 200nM

This first step comprised a 20µl reaction using USB PCR master mix (2x). The reaction was started by heating to 95 °C for 3 minutes followed by 40 cycles of PCR at 95 °C for 2 sec, 50 °C for 2 sec, and 72 °C at 2 sec. This step inhibited or prevented wild template amplification while the mutant templates were amplified. From step 1 dilute 1/500, 1 µl was transferred to step 2.

The second qPCR step uses the following components and conditions:
Internal for. primer: GAAAACACCGCAGCATGTC (SEQ ID NO.: 11) at 200 nM
Internal rev. primer: CTGCATGGTATTCTTTCTCTTCC (SEQ ID NO.: 12) at 200 nM; Mutant probe 858R: FAM-CAGATTTTGGCC GGGCCAAACTG-Phos (SEQ ID NO.: 7) at 200 nM; Mutant antiprobe: CAGTTTGGCCCGCCCAATATCTG-BHQ1 (SEQ ID NO.: 8) at 400 nM; Wild probe 858L: CalRed610-CAGATTTTGGGCTGACCAAACTG-Phos (SEQ ID NO.: 9) at 200 nM; Wild antiprobe: GCAGTTTGGCCAGCCCATAATCTG-BHQ2 (SEQ ID NO.; 10) at 400 nM
Cycling conditions: Real-time PCR was conducted as described in Example 1 except that the annealing/extension step was at 52 °C for 1 min.

Fig. 14 shows qPCR curves from two tubes containing the same reaction product from the first pre-amp step but with different probes, wild and mutant, in each tube. The positive (upward) curve shows the mutant detecting very early with exponential amplification starting at 9 cycles. The negative curve shows no detection of the wild template that originally was in abundance.

Example 11

One step iDDS and "Wild Terminator" method detecting EGFR Exon 21 mutants: The "Wild Terminator" probe and the iDDS probe were used together in a single real-time PCR reaction, the "Wild Terminator" probe inhibiting or blocking amplification of the wild templates and the iDDS probe detecting the mutant templates. The procedure would only work if the mutant frequency is about 1% or higher. In this example, 200 copies of the mutant template were used with 10,000 copies of the wild template (2% mutant). All primers and probes were the same as in Example 10 above, except the internal primers were used at 400 nM. The "Wild Terminator" probe was used at 150 nM, but similar results were seen with the "Wild Terminator"probe at 100 or 200 nM.

Cycling conditions: Real-time PCR was conducted as described in Example 1 with the annealing/extension step at 52 °C for 1 min.

Fig. 17 shows two curves from one tube containing the *"Wild Terminator"* probe and the wild and mutant iDDS probes for the L858R site in EGFR.

Example 12

ISAM isothermal amplification with ZIPR DDS probes and qPCR detection: DNA or RNA target sequences can be isothermally amplified and detected with a DDS primer-probe at one end and a primer with a 5' RNA polymerase promoter sequence at the other end. In this example, a T7 RNA polymerase promoter sequence was used. This method employs a reverse transcriptase to create a cDNA copy of the target region with the T7 site appended to the 5' end. Then a ZIPR DDS primer-probe served as a primer to copy the cDNA including the T7 site, thereby creating a double stranded product with a T7 promoter recognition sequence. A RNA polymerase, in this case a T7 RNA polymerase, then made RNA copies of the product that served as template for further amplification cycles, alternating between DNA and RNA products generated. RNaseH facilitated this process by degrading the RNA strand of an RNA:DNA hybrid. RNaseH can be provided separately, or as a reverse transcriptase enzyme with RNase function, to make cDNA copies of the RNA products.

A NUCLISENS.RTM Basic Kit (BioMerieux, Inc) was used. During amplification, the fluorescent-labeled ZIPR primer-probes were incorporated into the DNA products and were separated from any quencher-labeled antiprobes available, thereby providing qPCR detection. Due to the low temperature amplification, the antiprobes were made shorter. The probe and primer components were used as follows:
GAPDH-Cy3F1: Cy3- GAGTCAACGGATTTGGTCGT (SEQ ID NO.: 42) at 200nM; GAPDH-BHQ2: ATCCGTTGACTC-BHQ2 (SEQ ID NO.: 43) at 400nM; GAPDH-T7R1: AATTCTAATACGACTCACTATAGGGAGAAGGGACAAGCTTCCCGTTCTCAG (SEQ ID NO.: 44) at 200nM.

This test was performed with 1ng of GAPDH RNA as the starting template. The initial RT step was performed at 65 °C for 5 mins, and then 41 °C for 5 mins. Exponential ISAM was then performed using such as a qPCR machine or a water bath between 37 and 45 degrees, most typically at 40 °C to 42 °C. In one example, the qPCR step was run with two slightly different temperatures, cycling back and forth between 42 °C for 30 sec, and 40 °C for 30 sec, for 80 cycles. Fluorescent emissions were assessed at the second step per cycle (40 °C step).

In Fig. 18, the positive amplification curve shows detection of the ISAM qPCR DNA products with the ZIPR DDS primer-probe. The RNA products were not detected. Most of the exponential amplification phase was completed in the first 20 mins.

Example 13

ISAM isothermal amplification with internal DDS (iDDS) probes: ISAM amplification and qPCR detection can be performed as in Example 12 above, but using an internal DDS probe instead of a terminal primer-probe. In addition, both the forward and reverse primers could have a T7 sequence appended, a modification that increases the quantity of products generated about 20 to 50 percent. However, due to the low temperature used, the DDS probe and antiprobe were modified by using a long probe (24bp) and a shorter antiprobe (15bp). GAPDH-T7F1: AATTCTAATACGACTCACTATAGGGAGAAGGGAGTCAACGGATTTGGTCGT (SEQ ID NO.: 45) at 300nM; GAPDH-R1: AATTCTAATACGACTCACTATAGGGAGAAGGGACAAGCTTCCCGTTCTCAG (SEQ ID NO.: 46) at 300nM; GDH iDDS probe: FAM-CCTTCATTGACCTCAACTACATGG-amino (SEQ ID NO.: 47) at 150nM; GDH iDDS antiprobe: TGAGGTCAATGAAGG-BHQ1 (SEQ ID NO.: 48) at 300nM

The testing templates were GAPDH RNA and HIV RNA. Both the RT-PCR and the qPCR steps were performed as described in Example 13 except that qPCR cycling was for 60 one-minute cycles at one temperature, 41 °C, with fluorescent detection assessed every cycle. In Fig. 19, the positive amplification curve shows ISAM qPCR detection of the GAPDH DNA products with an internal DDS probe and antiprobe. The flat curve was from the HIV control template. With the internal DDS probe, detection was more stringent and thus the amplification curve rose more gradually.

Example 14

On-chip ISAM isothermal amplification and fluorescent detection: A chip array was hand-printed on CODELINK.RTM slides (GE Healthcare) with multiple spots that contained primers for either the GAPDH gene or the Rab9 gene. Those primers comprised from the 5′ end: an amino modification, a spacer, a T7 sequence, and a gene-specific reverse primer sequence. Following the CODELINK.RTM protocol, the primers were covalently joined to the printed slides via their 5′ amino modification. They were then treated to block non-specific binding, washed and dried. The spots were arranged in a semi-checker board pattern alternating between GAPDH and Rab9 primer spotting. The ISAM reaction was done on the chip, adding 150ng each of GAPDH RNA and Rab9 RNA, plus a Cy3-labeled GAPDH probe comprising a forward primer sequence, and a FAM-labeled Rab9 probe comprising a T7 sequence and a forward primer sequence.

The reaction was run under a coverslip, but in a sealed chamber, and maintained at 41 °C for at least 2 hrs in a water-bath. Chips were then washed with: 2xSSC/0.1% SDS,

.1xSSC/0.1% SDS, 1xSSC, then 0.01xSSC, then spun dry. Detection was made with a Perkin Elmer microarray scanner. The GDH and Rab9 specific primers and probes were: GDH-R1s: amino-spacer18-ATTTCTAATACGACTCACTATAGGGAGAAGGGACAAGCTTCCCGTTCTCAG (SEQ ID NO. 49); Rab9-R1s: amino-spacer18-ATTTCTAATACGACTCACTATAGGGAGAAGGAAATGGTGTCCTCAGGCTTC (SEQ ID NO. 50); GDHCy3F1: Cy3-GAGTCAACGGATTTGGTCGT (SEQ ID NO. 51) at 900nM; Rab9-FAM-T7F1: FAM-AATTCTAATACGACTCACTATAGGGAGAAGGCAATGGCAGGAAAATC (SEQ ID NO. 52) at 900nM

In Fig. 20, a pattern of green and blue spots was observed on the array indicating that on-chip ISAM gene specific amplification and detection could be performed with one primer attached to the chip. Due to low shading differences between the black background and the blue FAM-labeled Rab9 specific spots, those gene specific fluorescent dots were represented as white circles in the black/white figure shown. The green Cy3-labeled GAPDH specific spots can be more easily seen in the black/white figure as either solid white dots or white dots with a gray center. Amplification and detection was exclusive to each gene.

Example 15

Detecting a variable deletion mutant of EGFR exon 19 using two probe:antiprobe compositions on the same amplicon: In lung cancer and related disease, variable deletion mutants commonly occur in exon 19 of the EGFR gene involving the loss of 9 to 24 bases in the region comprising codons 746 to 753, and such deletions are the most common biomarker for responsiveness to tyrosine kinase inhibitors. These diagnostic deletions in exon 19 (known as Del-19) have generally been detected by sequencing methods. Dahse et al. 2008 has developed a PCR and gel-based assay that uses a special primer to bridge and amplify the most common 15 bp Del-19 mutant, however, this assay fails with other exon 19 deletion mutants. The example reported here overcomes this limitation using a pair of probe:antiprobe compositions in a qPCR assay to detect two aspects of an EGFR exon 19 amplicon. A primer-probe with one label is used to amplify and detect the terminal end of all amplicons of the targeted segment with or without a Del-19 mutant, and a second iDDS probe with a different label will detect an internal segment comprising codons 746 to 753 only if the wild type sequences are present. By subtraction, this two probe system thus discerns the relative proportion of Del-19 mutants to wild type. The primer-probe employed is a ZIPR probe:antiprobe that also serves as a forward primer: CalRed610-TCTGGATCCCAGAAGGTGAG (SEQ ID NO: 56) at 200 nM; CTCACCTTCTGGGTTCCAGA-BHQ2 (SEQ ID NO: 57) at 400 nM

The internal iDDS probe:antiprobe comprises: Fam-CAAGGAATTAAGAGAAGCAACATC–Phos (SEQ ID NO: 81) at 200 nM; GATGTTGCCTCTCTTAATTCCTTG-BHQ1 (SEQ ID NO: 82) at 400 nM

The flanking reverse primer comprises: CGTAGGCTTCATCGAGGATT (SEQ ID NO: 53) at 200 nM.

Sometimes a small quantity (~100nM) of an unlabeled forward primer (TCTGGATCCCAGAAGGTGAG, SEQ ID NO: 78) is also provided to reduce a strong ZIPR signal and bring it in balance with the iDDS signal to facilitate diagnostic interpretation of the Del-19 mutant frequency.

Real time PCR was conducted as described in Example 1 using artificial gene targets with and without a 9 bp or a 15 bp deletion or using patient samples with or without a known Del-19 mutant. If all templates are wild, the iDDS signal should be relatively equivalent to the ZIPR probe signal, but if Del-19 mutants are present, the iDDS signal, compared to the ZIPR probe signal, should drop significantly relative to mutant frequency. This expected result was observed in the templates and samples tested.

Fig. 21 shows curves from one tube using the two probes above and with 100% wild template. The ZIPR probe shows a positive curve detecting the targeted amplicon, and the iDDS probe shows an equivalent positive curve since all templates are wild. Fig. 22 shows a diminished iDDS curve relative to the ZIPR probe curve since the template is 50% mutant. Fig. 23 shows a positive ZIPR probe curve and a flat iDDS curve since the wild sequence is absent at the Del-19 target site. This assay thus provides an index of the Del-19 mutant frequency without detecting a specific mutant sequence. The ZIPR signal is consistently high with either wild or mutant templates, while the signal height of the iDDS probe varies in proportion to the frequency of the wild vs. mutant template regardless of the size or sequence of the mutant present.

Example 16

An iDDS probe:antiprobe assay to detect a single base mutant of EGFR exon 20 at codon 790 associated with acquired resistance to tyrosine kinase inhibitors used for cancer therapy:

Patients with non-small cell lung cancer that are responsive to tyrosine kinase inhibitors typically relapse after one year due to an EGFR mutation T790M in exon 20. This mutation is due to a C to T base pair change in the second letter of codon 790, causing a threonine to methionine missense substitution (ACG > ATG). Detecting such single base mutants thus provides a diagnostic indicator for a change in therapy. In this example, a qPCR assay employs an iDDS

probe:antiprobe to detect the presence of the 790M mutant sequence in an amplified EGFR template. The primers and probes comprise:

F-primer: GCATCTGCCTCACCTCCAC (SEQ ID NO: 83) at 200nM; R-primer: GTCTTTGTGTTCCCGGACAT (SEQ ID NO: 84) at 200nM

Probe: FAM-TGAGCTCCATGATGAGTTGCACG-Phos (SEQ ID NO: 85) at 200nM

Antiprobe: CGTGCAACTCTTCATGCAGCTCA-BHQ1 (SEQ ID NO: 86) at 400nM

Cycling conditions: Real-time PCR was conducted as described in Example 1. A positive curve indicates the 790M sequence is present.

Example 17

Multiplex assay to detect influenza A or B based on two ZIPR probe:antiprobe compositions:

This assay employs two ZIPR probes with different fluorescent labeling to detect either influenza A or influenza B in a sample comprising:

F-primer: CTTCTAACCGAGGTCGAAACGTA (SEQ ID NO: 87) at 200nM

A-Probe: Fam-GCTTTGAGGGGGCCTGA (SEQ ID NO: 88) at 200nM

A-Antiprobe: TCAGCCCCCCTCAAAGC-BHQ-1 (SEQ ID NO: 89) at 400nM

R-primer: CTAATTGTCTCCCTCTTCTGGTGA (SEQ ID NO: 90) at 200nM

B-Probe: CalRed610-CCCAATTTGGTCAAGAGCAC (SEQ ID NO: 91) at 200nM

B-Antiprobe: GTGCTGATGACCAAATTGGG-BHQ-2 (SEQ ID NO: 92) at 400nM

Cycling conditions: Real-time PCR was conducted as described in Example 1. A FAM-positive curve indicates flu A is present and a CalRed610 positive curve indicates flu B is present.

Example 18

Multiplex assay to detect variable mutations in KRAS exon 1, codons 12 and 13, associated with reduced response to EGFR targeted therapies in lung and colon cancer patients.

This assay is similar to the Del-19 detection scheme and employs a non-specific ZIPR probe system with HEX fluorescent labeling and a wild type iDDS probe system with FAM labeling.

The iDDS probe comprises:

FAM-CCTACGCCACCAGCTC-Phos (SEQ ID NO. 93) at 200 nM

GAGGTGGTGGCGTAGG-BHQ1 (SEQ ID NO. 94) at 400 nM

The ZIPR probe comprises:

HEX-TGGATCATATTCGTCCACAAAA (SEQ ID NO. 95) at 200 nM

TTTTGAGGACGAATATGATCCA-BHQ1(SEQ ID NO. 96) at 400 nM

Flanking primer is: CCTGCTGAAAATGACTGAATATAAA (SEQ ID NO. 97) at 200 nM

CLAIMS:

1. A DNA Detection Switch (DDS) probe system for selectively detecting a target nucleotide sequence, comprising:

a probe:antiprobe detection system comprising;

(a) a probe oligonucleotide comprising a nucleotide sequence complementary to a first target nucleotide sequence, and a first fluorescence emitter attached thereto; and

(b) an antiprobe oligonucleotide comprising a nucleotide sequence fully complementary to the probe oligonucleotide except for at least one mismatched base in a non-terminal position, and a fluorescence modulator, wherein the fluorescence modulator is attached to the antiprobe oligonucleotide or is a region of the antiprobe oligonucleotide,

wherein:

(i) the probe oligonucleotide is configured for greater hybridization affinity for the first target nucleotide sequence than for the antiprobe oligonucleotide; and the antiprobe oligonucleotide is configured for greater hybridization affinity for the probe oligonucleotide than for a second target nucleotide sequence of interest;

(ii) in the presence of the first target nucleotide sequence, the probe oligonucleotide and the first target nucleotide sequence form a duplex, whereupon the first fluorescence emitter and the fluorescence modulator do not interact, thereby providing a first detectable signal;

(iii) in the absence of the first target nucleotide sequence, the probe oligonucleotide and the antiprobe oligonucleotide form a duplex, thereby allowing the first fluorescence emitter and the fluorescence modulator to interact to provide a modulated detectable signal, wherein the first detectable signal and the modulated detectable signals are distinguishable; and

(iv) in the presence of the second target nucleotide sequence differing from the first target nucleotide sequence by at least one base mismatch, the probe oligonucleotide and the antiprobe oligonucleotide preferentially form a duplex, thereby inhibiting the probe oligonucleotide from forming a duplex with the second target nucleotide sequence.

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2. The DDS probe system of claim 1, wherein the duplex of the probe oligonucleotide and the first target nucleotide sequence, and the duplex of the probe oligonucleotide and the antiprobe oligonucleotide differ by at least 2 kcal/mol in ΔG and at least 4°C in T_m; and wherein the duplex of the probe oligonucleotide and the second target nucleotide sequence, and the duplex of the probe oligonucleotide and the first target nucleotide sequence differ by at least 4 kcal/mol in ΔG and at least 8°C in T_m.

3. The DDS probe system of claim 1 or 2, wherein the probe oligonucleotide sequence is configured with a mismatched base position two bases away from an expected mismatch between the second target nucleotide sequence and the sequence of the probe; whereby said two mismatched bases generate an internal two or three base non-hybridized region of the probe oligonucleotide:second target nucleotide sequence duplex, said duplex thereby having a ΔG and T_m less than the ΔG and T_m of the probe oligonucleotide:antiprobe oligonucleotide duplex.

4. The DDS probe system of any one of claims 1 to 3, wherein the probe oligonucleotide is attached to a solid substrate.

5. The DDS probe system of any one of claims 1 to 4, wherein the fluorescence modulator is selected from the group consisting of: a quencher compound, a fluorescent compound, a metallic particle, and a guanine-rich conjugate.

6. The DDS probe system of any one of claims 1 to 5, wherein at least one of the probe oligonucleotide and the antiprobe oligonucleotide comprises at least one of: a non-natural nucleotide, a minor groove binder (MGB), and a Zip nucleic acid (ZNA).

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7. The DDS probe system of any one of claims 1 to 6, wherein said probe:antiprobe detection system is an iDDS probe system, wherein the 3' end of the probe oligonucleotide is blocked to prevent polymerase extension; and wherein the system further comprises a pair of flanking primers configured for amplifying a nucleic acid region comprising the first target nucleotide sequence.

8. The DDS probe system of any one of claims 1 to 6, wherein said probe:antiprobe detection system is an iDDS probe system, wherein the 3' end of the probe oligonucleotide, and the antiprobe oligonucleotide, is blocked to prevent polymerase extension; and wherein the system further comprises a pair of flanking primers configured for amplifying a nucleic acid region comprising the first target nucleotide sequence.

9. The DDS probe system of any one of claims 1 to 6, wherein said probe:antiprobe detection system is a Flip probe system, wherein the antiprobe oligonucleotide further comprises, at the 3′ end thereof, a first primer oligonucleotide and the system further comprises a second primer oligonucleotide, wherein the first and the second primer oligonucleotides are configured for amplifying a nucleic acid region comprising the first target nucleotide sequence.

10. The DDS probe system of claim 9, wherein the antiprobe oligonucleotide and the first primer oligonucleotide are linked by an abasic spacer region.

11. The DDS probe system of any one of claims 1 to 6, wherein said probe:antiprobe detection system is a ZIPR probe system, wherein the nucleotide sequence of the probe oligonucleotide comprises a primer sequence and is configured to cooperate with a primer oligonucleotide to amplify the target nucleotide sequence, whereupon a detectable signal is generated when the probe oligonucleotide is incorporated into an amplicon.

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12. The DDS probe system of claim 11, further comprising an additional probe oligonucleotide, wherein said probe oligonucleotide and said additional probe oligonucleotide selectively hybridize to different target nucleotide sequences of a nucleic acid template; and wherein the additional probe oligonucleotide has a second flluorescence emitter attached thereto, and wherein the first fluorescence emitter and the second fluorescence emitter provide two different detectable signals or the same detectable signal.

13. The DDS probe system of claim 12, wherein the probe oligonucleotide comprises a primer-probe that is configured for cooperating with the primer oligonucleotide to amplify a first amplicon with the first fluorescence emitter, thereby providing a signal relative to amplicon frequency, and wherein the additional probe oligonucleotide is either a second primer-probe or an internal probe that comprises the second fluorescence emitter and a sequence complementary to a second target nucleotide sequence comprising a variable sequence segment of the first amplicon, or a variable sequence elsewhere in a nucleic acid template; whereupon the difference in signaling between the probe oligonucleotide and the additional probe oligonucleotide provides an indicator of the frequency of the variable sequence relative to the frequency of the first amplicon.

14. The DDS probe system of any one of claims 1 to 13, said system further comprising a G-Force primer-probe wherein the probe oligonucleotide and the antiprobe oligonucleotide are configured as a one-component primer-probe oligonucleotide to amplify and label a segment of the first target nucleotide sequence and comprises sequentially: (i) the first fluorescence emitter attached to the probe oligonucleotide at the 5' end, (ii) a cytosine-rich oligonucleotide segment of 7 to 9 bases, (iii) an abasic spacer, (iv) the antiprobe oligonucleotide comprising a guanine-rich segment complementary to the cytosine-rich oligonucleotide segment, and (v) a 3' primer sequence complementary to one end of the first target nucleotide sequence; whereupon, when the primer-probe oligonucleotide is

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incorporated into the amplicons generated, a detectable signal from the first fluorescence emitter is enabled.

15. The DDS probe system of any one of claims 1 to 14, further comprising an isothermal amplification system suitable for amplifying and detecting a RNA or DNA target sequence comprising a RNA polymerase promoter enzyme, a reverse transcriptase enzyme and an RNaseH enzyme, and comprising at least one primer-probe oligonucleotide configured to cooperate with a flanking primer to amplify the first target nucleotide sequence wherein: at least one primer sequence comprising the primer-probe oligonuceotide or the flanking primer further comprises a 5' RNA polymerase promoter sequence; whereupon signaling and transcription is provided from one or both ends of an amplicon.

16. The DDS probe system of any one of claims 1 to 14, further comprising an isothermal amplification system suitable for amplifying and detecting a RNA or DNA target sequence comprising a RNA polymerase promoter enzyme, a reverse transcriptase enzyme and an RNaseH enzyme, and comprising at least one primer-probe oligonucleotide configured to cooperate with a flanking primer to amplify the first target nucleotide sequence wherein: at least one primer sequence comprising the primer-probe oligonuceotide or the flanking primer further comprises a 5' RNA polymerase promoter sequence; and one of the primer-probe oligonucleotides or flanking primer is affixed to a solid substrate; and wherein the antiprobe oligonucleotide comprises a sequence complementary to the RNA polymerase promoter sequence; whereupon signaling and transcription is provided from one or both ends of an amplicon.

17. The DDS probe system of claim 7 or 8, further comprising an isothermal amplification system suitable for amplifying and detecting a RNA or DNA target sequence comprising a RNA polymerase promoter enzyme, a reverse transcriptase enzyme, a RNaseH enzyme and the pair of flanking primers wherein one or both of the flanking primers in the pair further comprises a 5' RNA polymerase

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promoter sequence wherein the probe oligonucleotide is complementary to an internal target sequence comprising 20 to 25 nucleotides in length; and the antiprobe oligonucleotide comprises 10 to 15 nucleotides in length.

18. The DDS probe system of claim 7 or 8, further comprising an isothermal amplification system suitable for amplifying and detecting a RNA or DNA target sequence comprising a RNA polymerase promoter enzyme, a reverse transcriptase enzyme, a RNaseH enzyme and the pair of flanking primers of flanking primers wherein one or both of the flanking primers in the pair further comprises a 5' RNA polymerase promoter sequence and one of the flanking primers in the pair is affixed to a solid substrate; wherein the probe oligonucleotide is complementary to an internal target sequence comprising 20 to 25 nucleotides in length; and the antiprobe oligonucleotide comprises 10 to 15 nucleotides in length.

19. The DDS probe system of claim 7 or 8, wherein the system is configured to detect a variable nucleotide base position of exon 21 of the EGFR gene, said system comprising the oligonucleotides SEQ ID NOs.: 7-12, and 39-41.

20. The DDS probe system of claim 13, wherein the system is configured to detect a variable multi-base deletion within codons 746 to 753 of exon 19 of the EGFR gene, said system comprising at least one unlabeled primer, and two probe:antiprobe systems with different labeling; wherein the first probe:antiprobe system comprises a primer-probe complementary to a non-specific first sequence; wherein the second probe:antiprobe system is complementary to the wild-type sequence at the exon 19 deletion site and inhibits or excludes detection of target templates comprising a multi-base deletion within codons 746 to 753 of exon 19 of the EGFR gene; whereupon the presence and frequency of an exon 19 deletion is assessed by comparing the relative signaling of the two probe systems.

21. The DDS probe system of claim 20, wherein a primer set for the first probe:antiprobe system is SEQ ID NOs.: 56, 57 and 53, or SEQ ID NOs.: 79, 80

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and 12; and wherein a primer set for the second probe:antiprobe system is SEQ ID NOs.: 81, 82, and 53, or SEQ ID NOs.: 54, 55 and 53.

22. The DDS probe system of claim 21, wherein both the primer set for the first probe:antiprobe system and the primer set for the second probe:antiprobe system include supplemental primer SEQ ID NO.: 78.

23. The DDS probe system of any one of claims 1 to 22, wherein the antiprobe oligonucleotide is combined with a Taqman® or Molecular Beacon probe assay; wherein the antiprobe oligonucleotide comprises the fluorescence modulator and comprises a sequence partially complementary to a Taqman® or Molecular Beacon probe.

24. The DDS probe system of any one of claims 1 to 23, wherein the first target nucleotide sequence is a nucleotide variant of exon 19, 20 or 21 of the *EGFR* gene, a *VKORC1* gene, a *CYP2C9* gene, a *uidA* gene of E. coli, a gram positive bacterium 16s gene, a gram negative bacterium 16s gene, a mycobacterium inhA gene, a mycobacterium *rpoB* gene, a mycobacterium *16S* gene, a hemagglutinin (HA) gene of influenza virus, a matrix (M) gene of influenza A virus, a non-structural (NS) gene of influenza B virus, or a KRAS gene.

25. The DDS probe system of any one of claims 1 to 24, wherein the probe oligonucleotide and the antiprobe oligonucleotide comprises the nucleic acid sequences selected from the group consisting of: SEQ ID NOs.: 1 and 2, SEQ ID NOs.: 3 and 4, SEQ ID NOs.: 7 and 8, SEQ ID NOs.: 9 and 10, SEQ ID NOs.: 13 and 14, SEQ ID NOs.: 17 and 18, SEQ ID NOs.: 19 and 20, SEQ ID NOs.: 23 and 24, SEQ ID NOs.: 36 and 37, SEQ ID NOs.: 54 and 55, SEQ ID NOs.: 56 and 57, SEQ ID NOs.: 64 and 65, SEQ ID NOs.: 66 and 67, SEQ ID NOs.: 70 and 71, SEQ ID NOs.: 72 and 73, SEQ ID NOs.: 79 and 80, SEQ ID NOs.: 81 and 82, SEQ ID NOs.: 85 and 86, SEQ ID NOs.: 88 and 89, and SEQ ID NOs.: 91 and 92.

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26. The DDS probe system of any one of claims 1 to 25, further comprising an unlabeled blocking terminator probe for selectively suppressing qPCR amplification and detection of the first target nucleotide sequence versus the second target nucleotide sequence in a qPCR assay; wherein the terminator probe comprises: (i) an oligonucleotide complementary to the first target nucleotide sequence, (ii) a 5' end modified to resist exonuclease digestion, (iii) a 3' end modified to resist polymerase extension, and (iv) wherein the terminator probe is further configured with a Tm and ΔG that exceeds the Tm and ΔG of the primers and or probes of the qPCR assay by at least 5 kcal/mol in ΔG and by at least 5°C in Tm.

27. The DDS probe system of claim 26, to enhance binding, wherein the terminator probe further comprises at least one of a non-natural nucleotide, a minor groove binder (MGB), and a Zip nucleic acid (ZNA).

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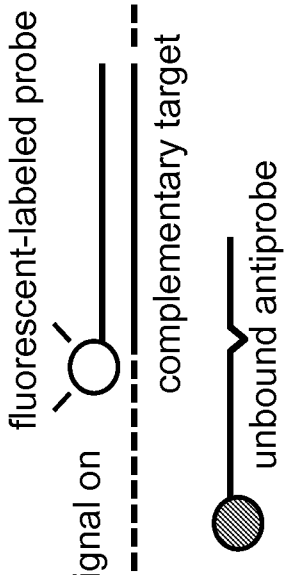


Fig. 1A

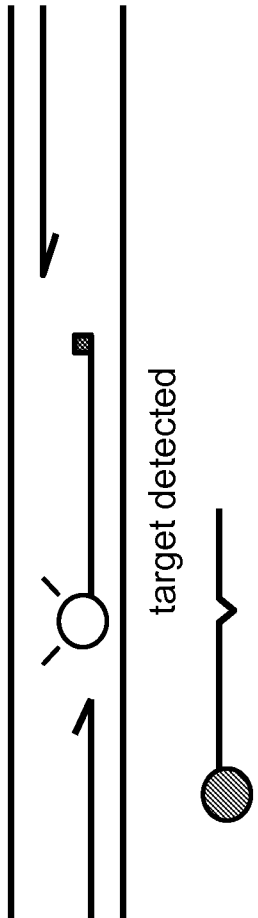


Fig. 1B

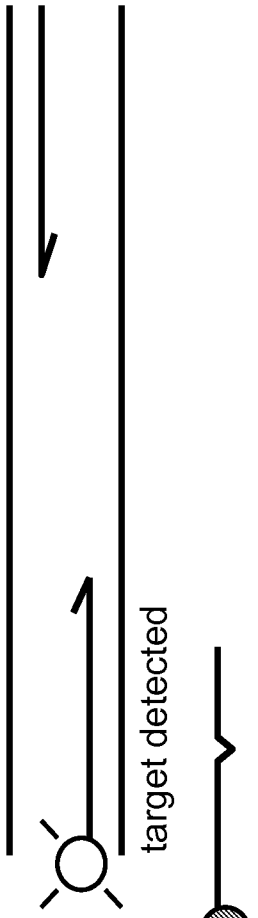


Fig. 1C

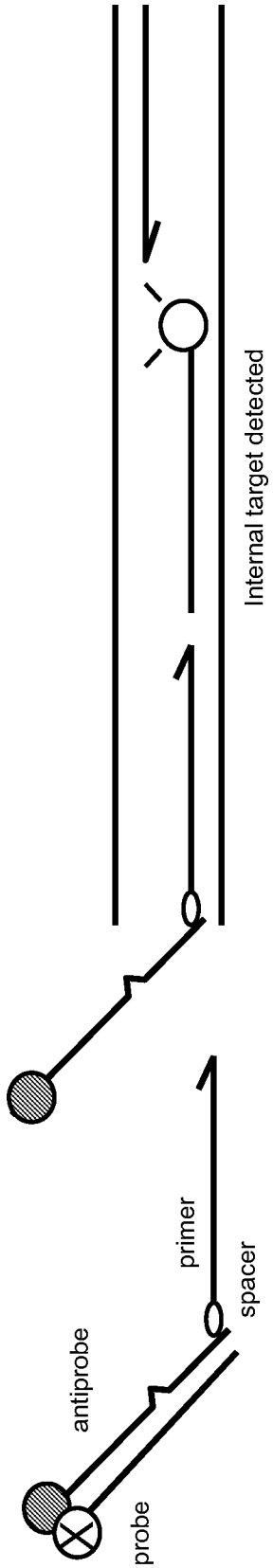


Fig. 2A

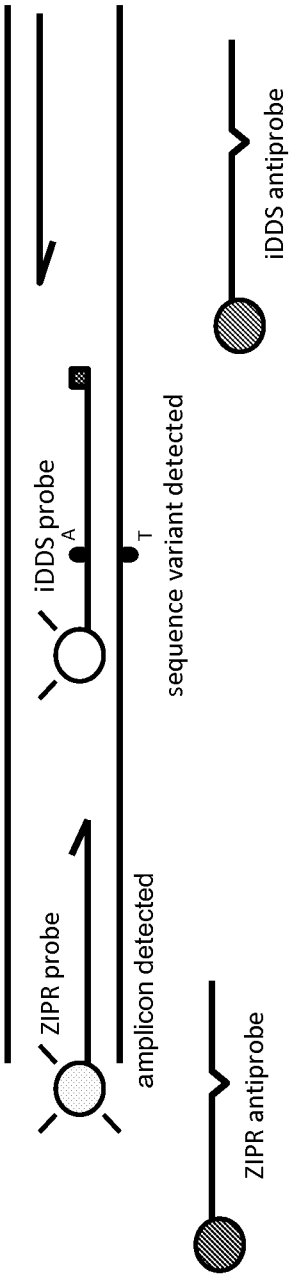


Fig. 2B

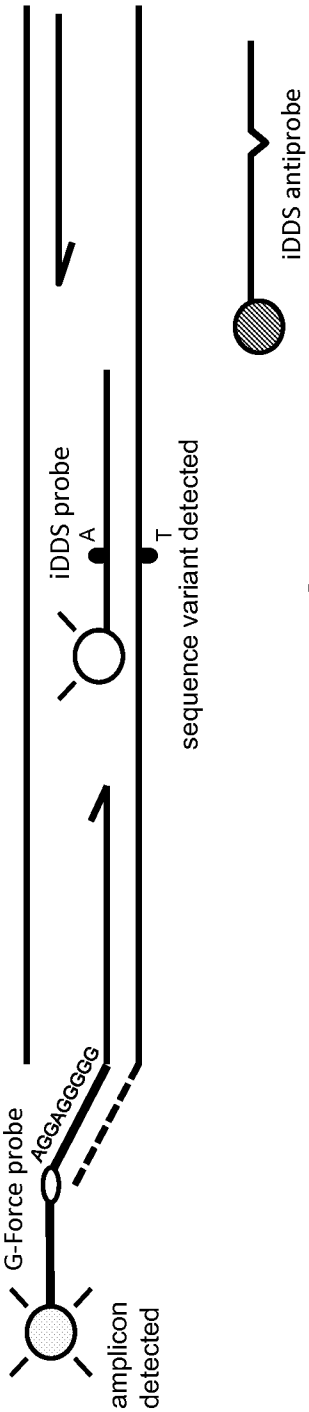
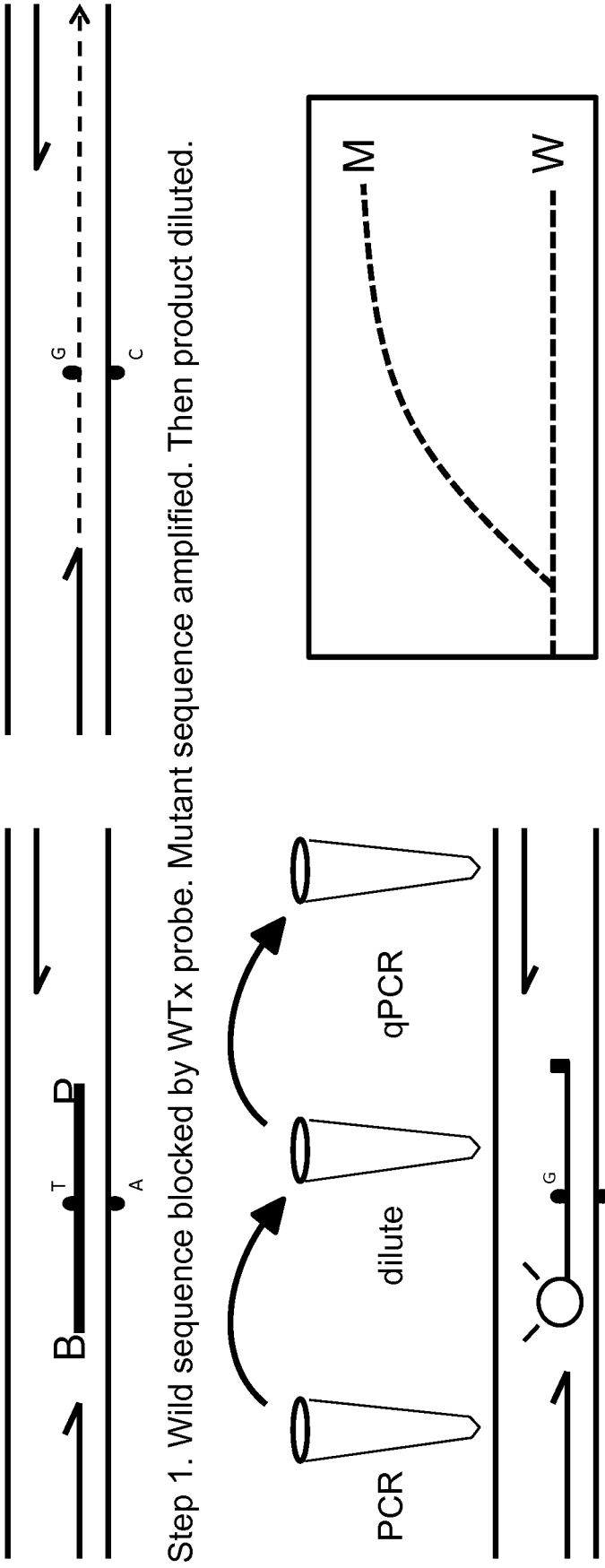


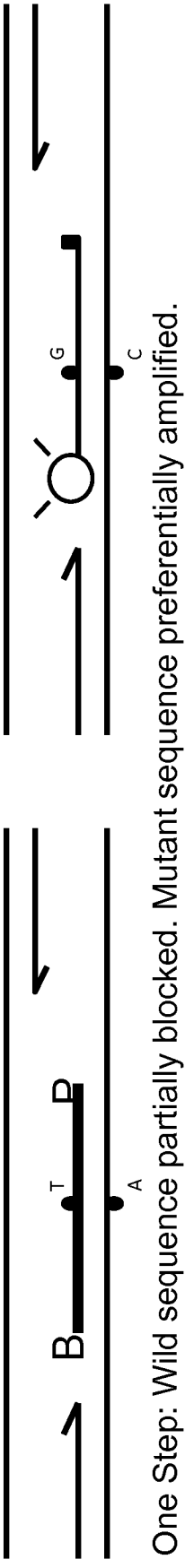
Fig. 2C



Step 1. Wild sequence blocked by WTx probe. Mutant sequence amplified. Then product diluted.

Step 2. qPCR: early amplification and detection of mutant variant, while wild type blocked.

Fig. 3A



One Step: Wild sequence partially blocked. Mutant sequence preferentially amplified.

Fig. 3B

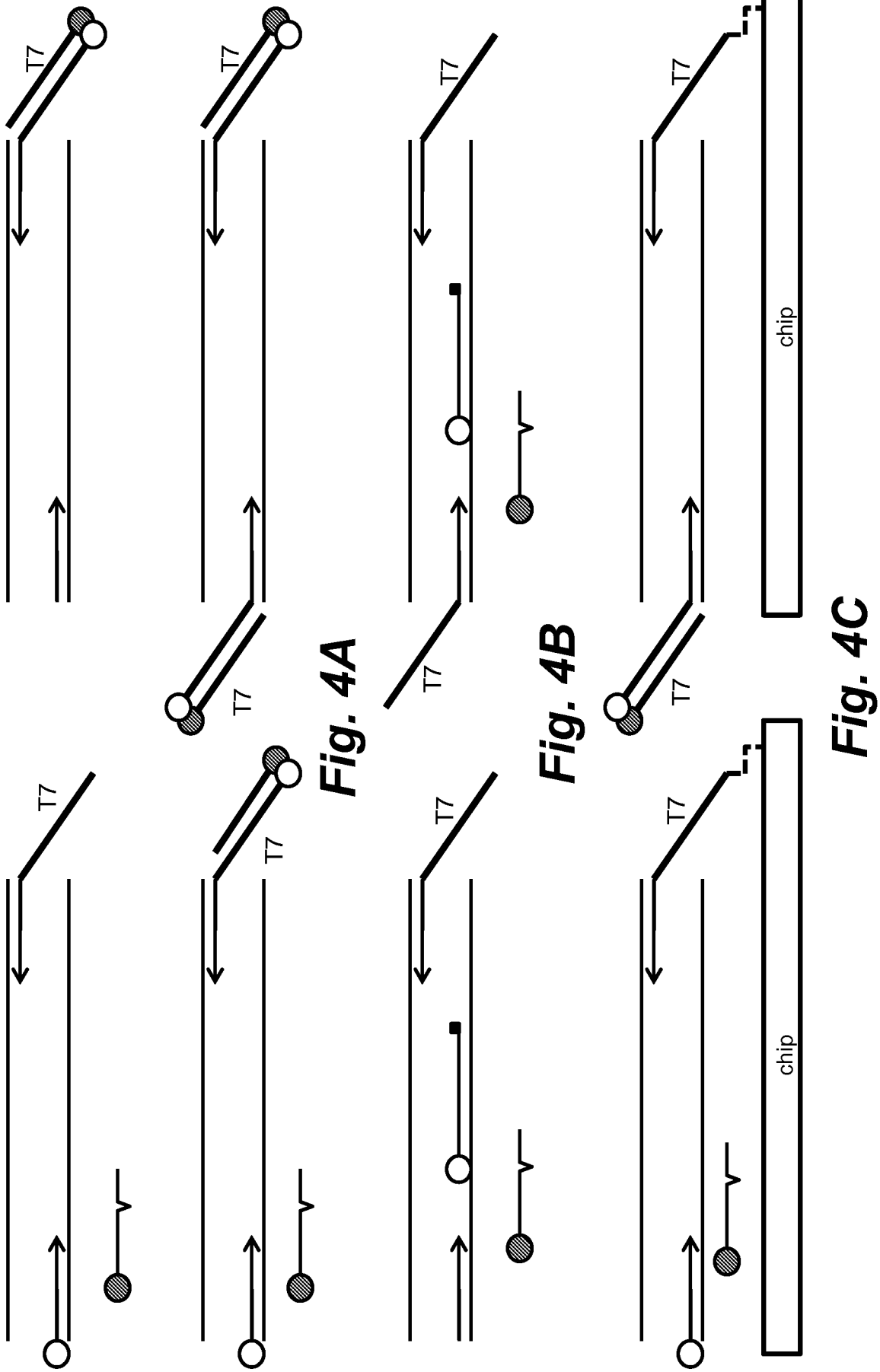


Fig. 4A

Fig. 4B

Fig. 4C

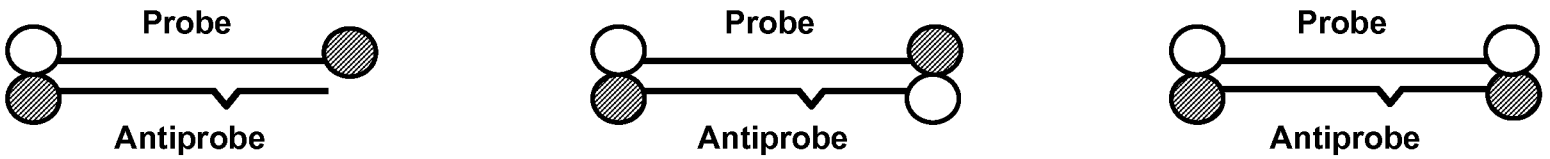


Fig. 5

5′-Label-CGCACC**C**GGCCAATG-Phos-3′ **(SEQ ID NO.: 1)**
ACTCGGTGGCGTGG**G**CCGGTTACCAACAA **(SEQ ID NO.: 58)**

VKORC1 SNP site
Probe: WildTarget duplex
Maximum Tm & ΔG

5′-Label-CGCACC**C**GGCCAATG-Phos-3′ **(SEQ ID NO.: 1)**
3′-Quencher- GCGTGG**G**CCG TTAC-5′ **(SEQ ID NO.: 2)**
C

Probe:Antiprobe duplex
Tm & ΔG reduced
Tm: -6.2 °C
ΔG: -2.4 kcal/mol

C
5′-label- CGCACC GGCCAATG-Phos-3′ **(SEQ ID NO.: 1)**
ACTCGGTGGCGTGG**A**CCGGTTACCAACAA **(SEQ ID NO.: 59)**

Probe:Variant Target duplex
Tm & ΔG reduced further
Tm: -5.7 °C
ΔG: -2.1 kcal/mol

Fig. 6

EGFR Forward primer SEQ ID NO.: 11
5′-TGAAACACCGCAGCATGTCAAGATCACAGATTTTGGGCGGGCCAAACTGCTGGGTGC
3′-ACTTTTGTCCGCTCGTACAGTTCTAGTGTCTAAAACCCGCCCGGTTTGACGACCCACG
GGAAGAGAAAGAATACCATGCAGAA-3′
CCTTCTCTTTCTTATGGTACGTCTT-5′
SEQ ID NO.: 60
MUTANT
SEQ ID NO.: 61
Reverse primer SEQ ID NO.: 12
EGFR Forward primer SEQ ID NO.: 11
5′-TGAAACACCGCAGCATGTCAAGATCACAGATTTTGGGCTGGCCAAACTGCTGGGTGC
3′-ACTTTTGTCCGCTCGTACAGTTCTAGTGTCTAAAACCCGACCGGTTTGACGACCCACG
GGAAGAGAAAGAATACCATGCAGAA-3′
CCTTCTCTTTCTTATGGTACGTCTT-5′
SEQ ID NO.: 62
WILD
SEQ ID NO.: 63
Reverse primer SEQ ID NO.: 12

Fig. 7A

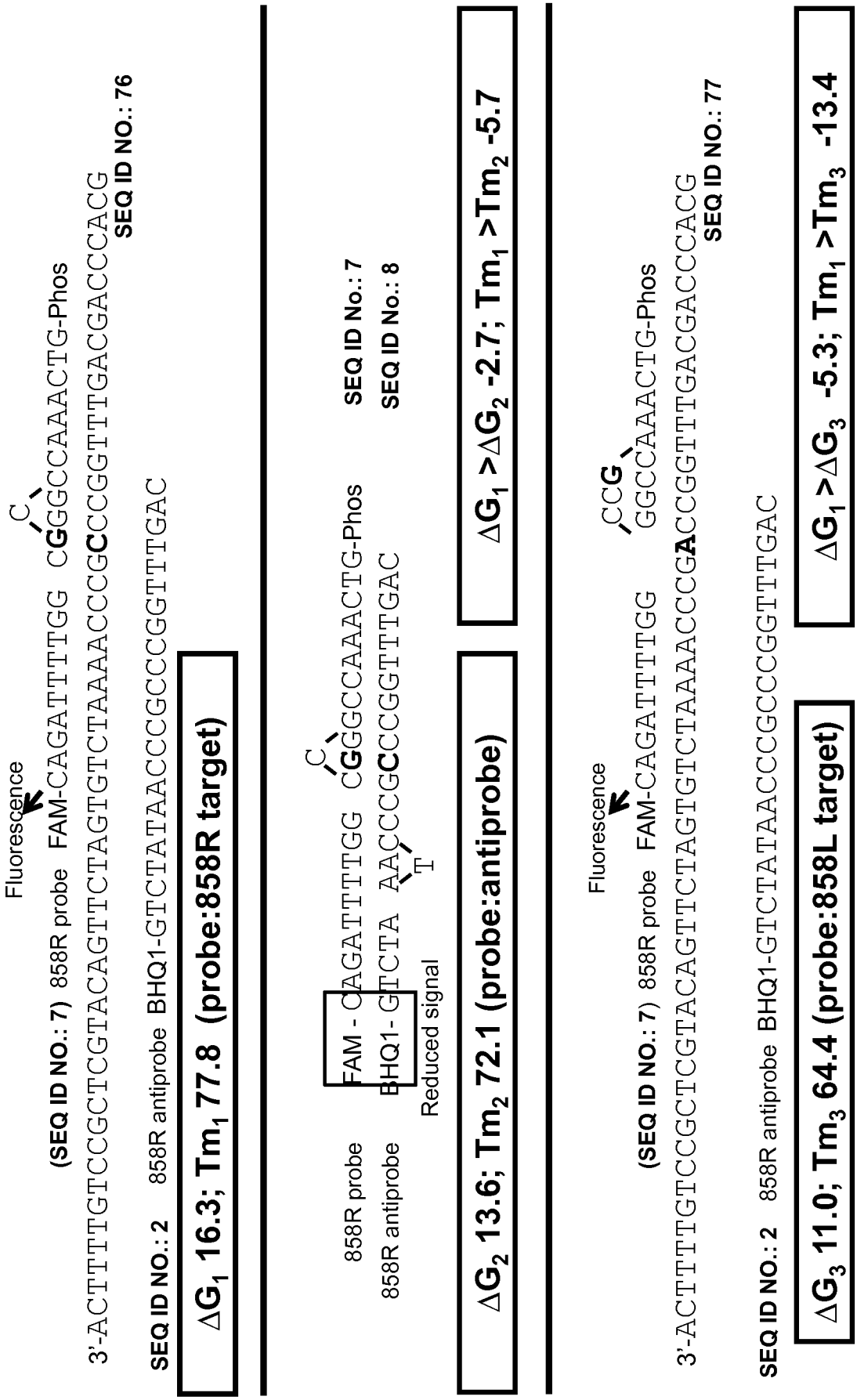

Fluorescence
(SEQ ID NO.: 7) 858R probe FAM-CAGATTTTGG CGGGCCAAACTG-Phos
3'-ACTTTTGTCCGCTCGTACAGTTCTAGTGTCTAAAACCCGCCCGGTTTGACGACCCACG
SEQ ID NO.: 76
SEQ ID NO.: 2 858R antiprobe BHQ1-GTCTATAACCCGCCCGGTTTGAC
ΔG1 16.3; Tm1 77.8 (probe:858R target)
858R probe FAM - CAGATTTTGG CGGGCCAAACTG-Phos
858R antiprobe BHQ1- GTCTA AACCCGCCCGGTTTGAC
SEQ ID No.: 7
SEQ ID No.: 8
Reduced signal
ΔG2 13.6; Tm2 72.1 (probe:antiprobe)
ΔG1 >ΔG2 -2.7; Tm1 >Tm2 -5.7
Fluorescence
(SEQ ID NO.: 7) 858R probe FAM-CAGATTTTGG CCGGGCCAAACTG-Phos
3'-ACTTTTGTCCGCTCGTACAGTTCTAGTGTCTAAAACCCGACCGGTTTGACGACCCACG
SEQ ID NO.: 77
SEQ ID NO.: 2 858R antiprobe BHQ1-GTCTATAACCCGCCCGGTTTGAC
ΔG3 11.0; Tm3 64.4 (probe:858L target)
ΔG1 >ΔG3 -5.3; Tm1 >Tm3 -13.4

Fig.7B

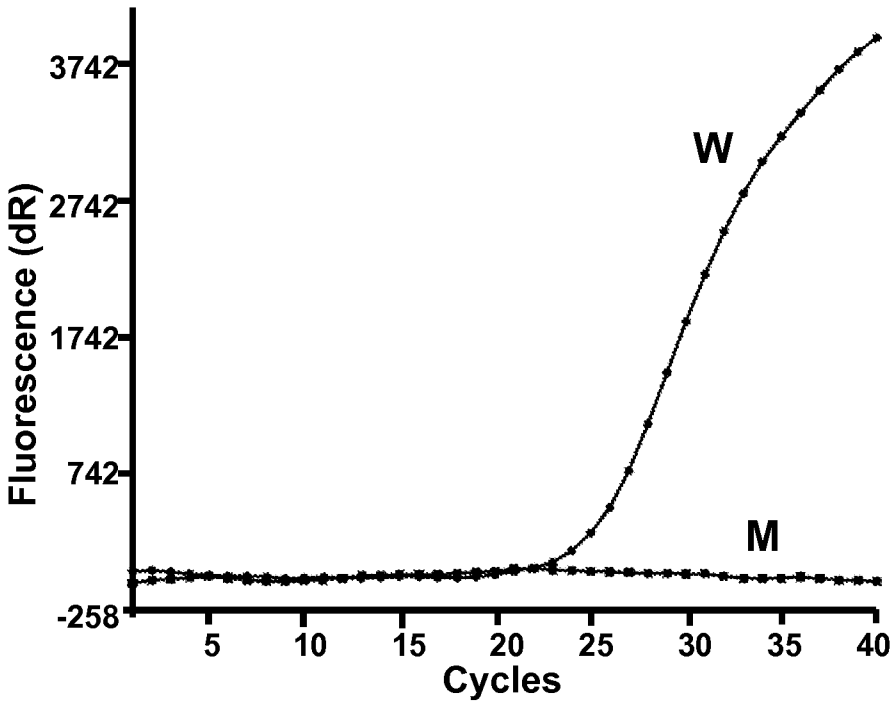
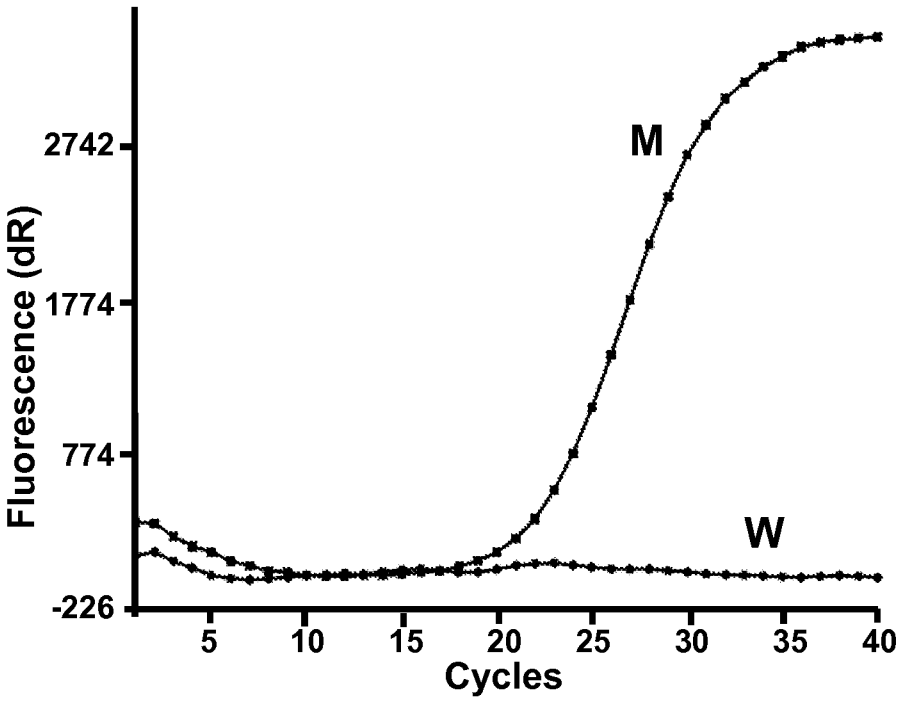

3742
2742
1742
742
-258
Fluorescence (dR)
W
M
5
10
15
20
25
30
35
40
Cycles

Fig. 8A


2742
1774
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Fluorescence (dR)
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W
5
10
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20
25
30
35
40
Cycles

Fig. 8B

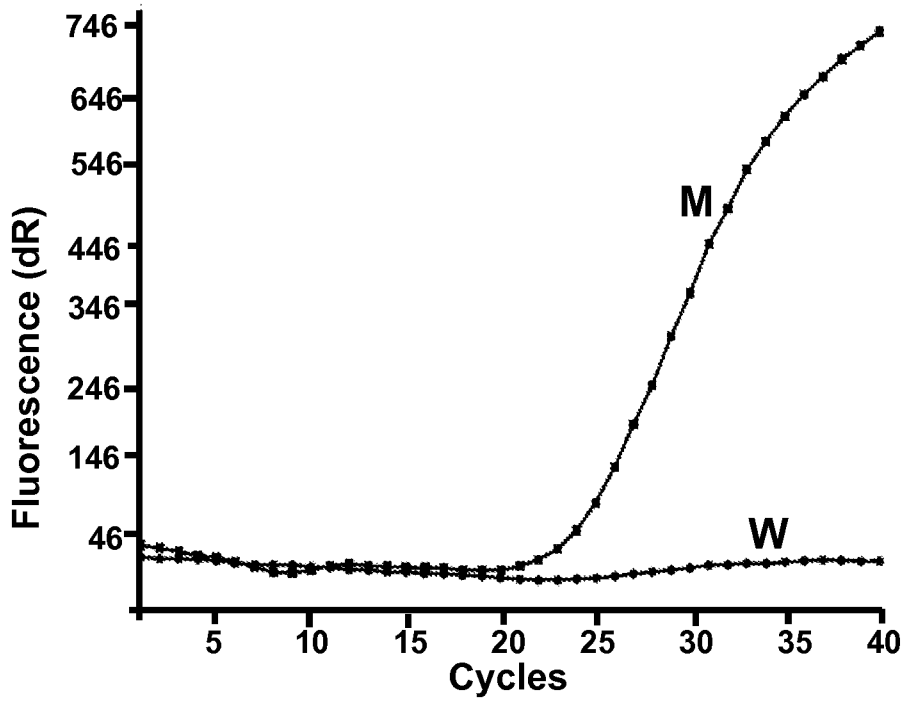

746
646
546
446
346
246
146
46
Fluorescence (dR)
M
W
5
10
15
20
25
30
35
40
Cycles

Fig. 9

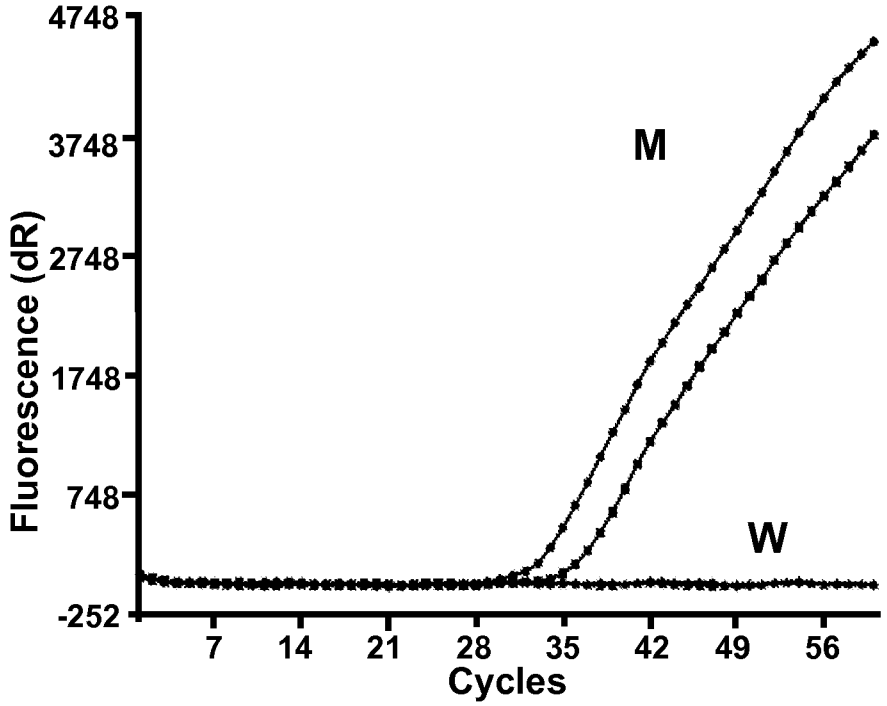


Fig. 10A

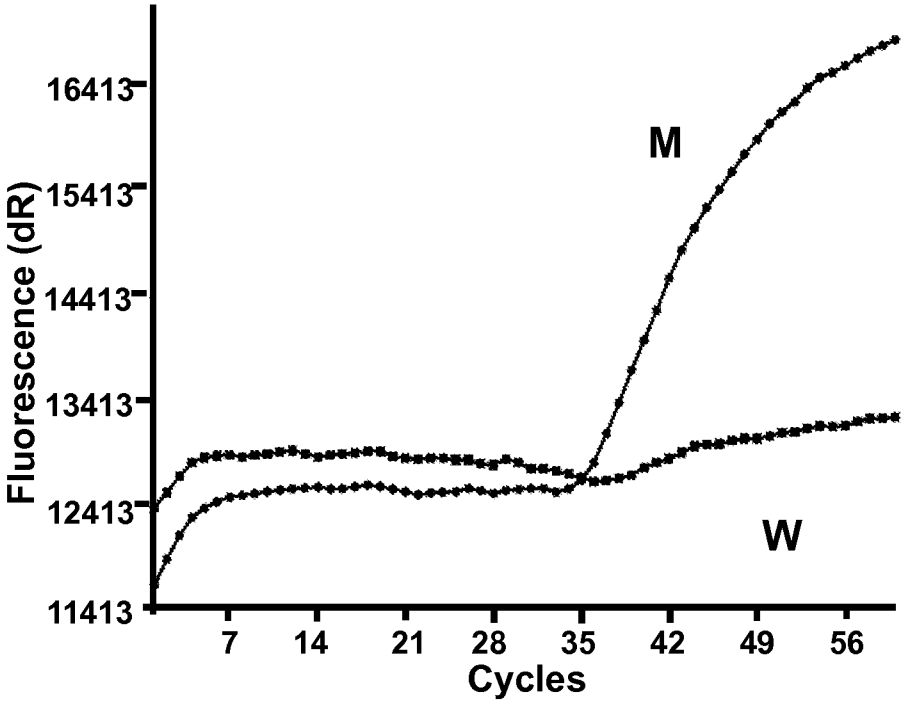


Fig. 10B

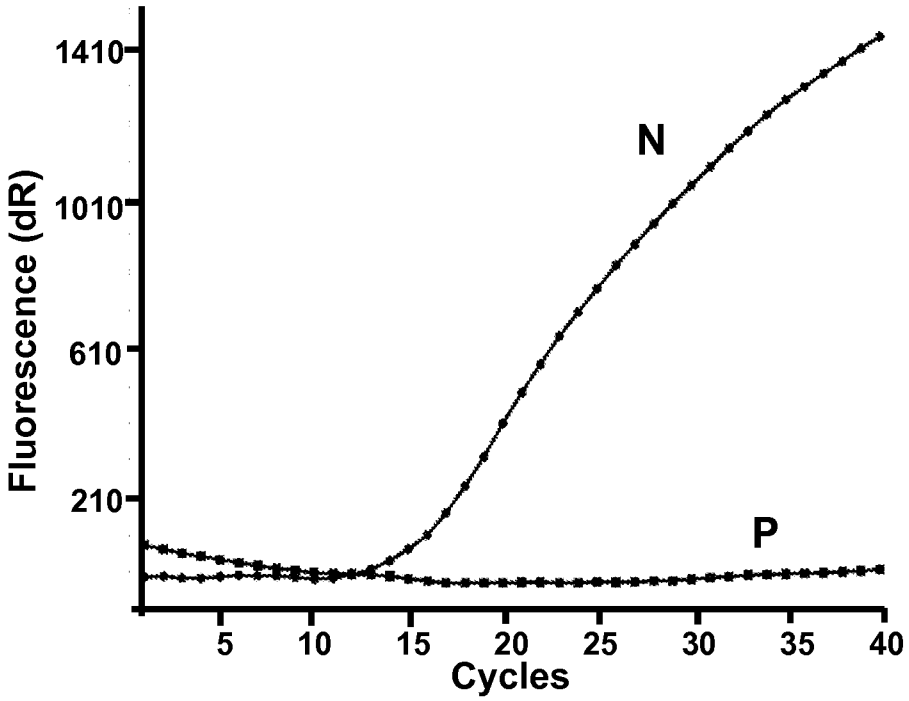


Fig. 11A

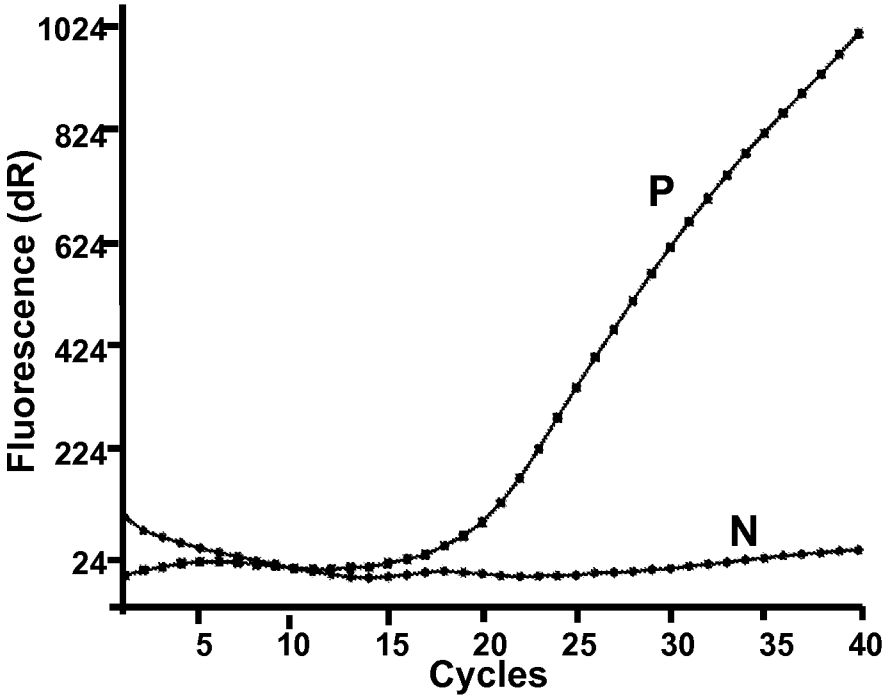


Fig. 11B

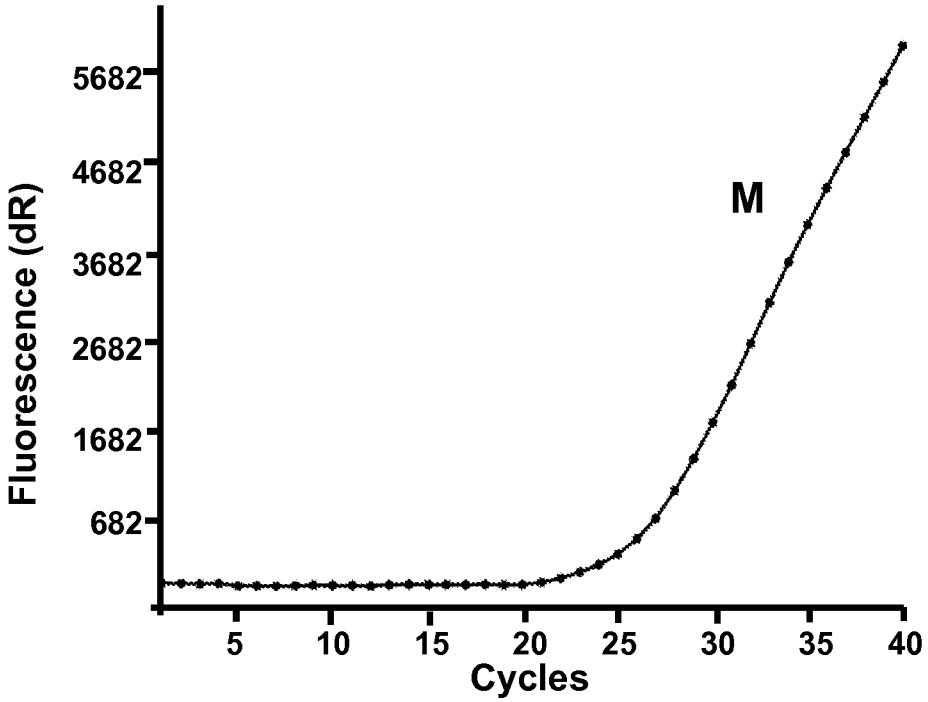

5682
4682
3682
2682
1682
682
Fluorescence (dR)
M
5
10
15
20
25
30
35
40
Cycles

Fig. 12

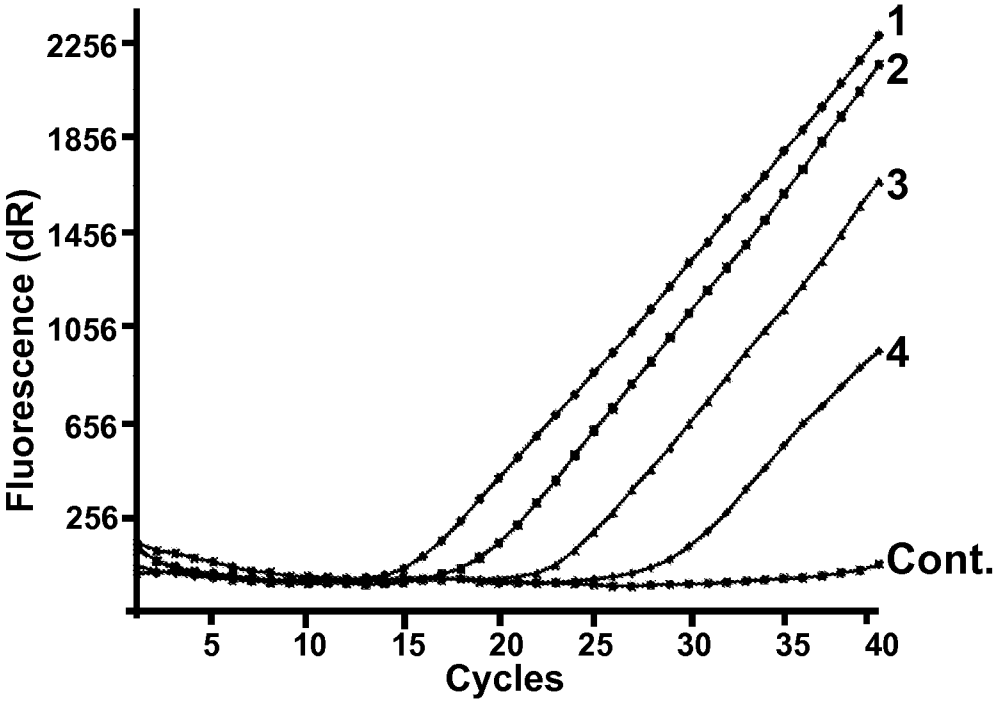


Fig. 13A

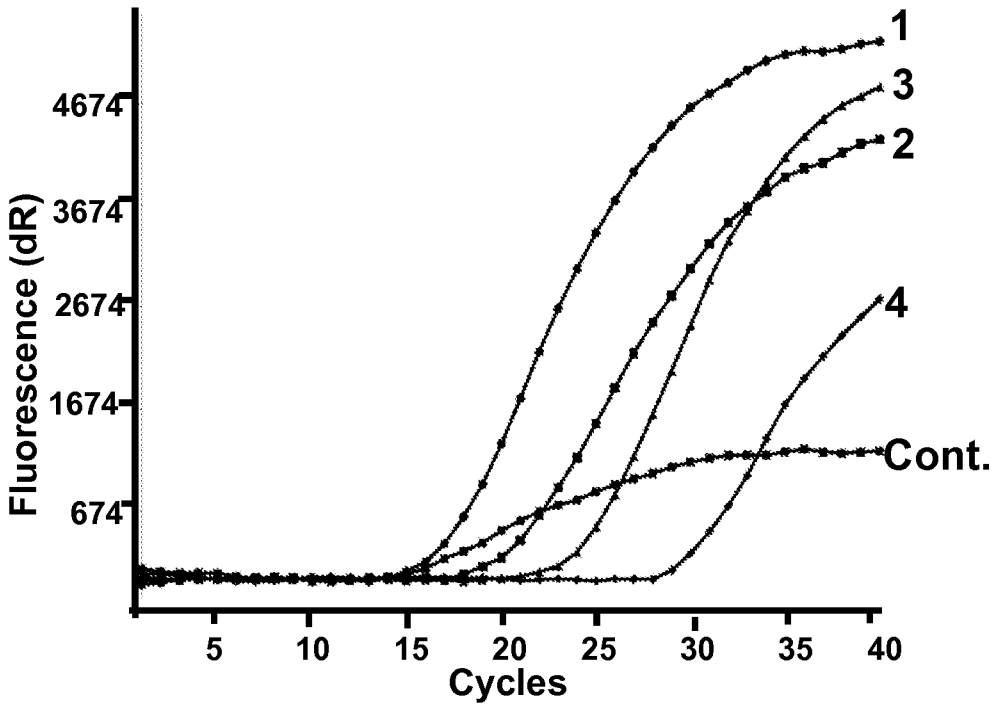


Fig. 13B

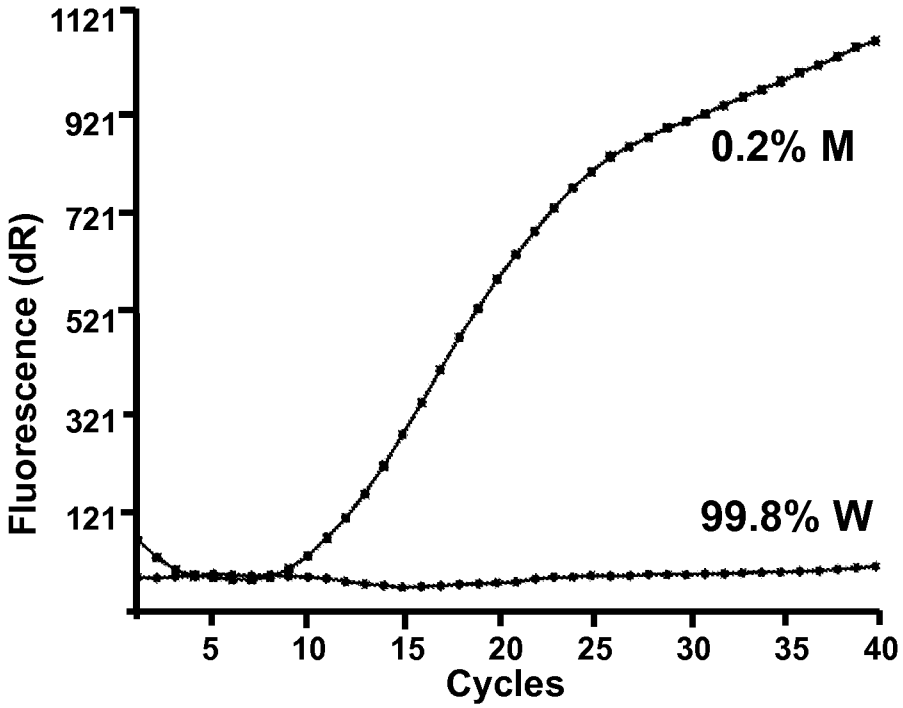

1121
921
721
521
321
121
Fluorescence (dR)
0.2% M
99.8% W
5
10
15
20
25
30
35
40
Cycles

Fig. 14

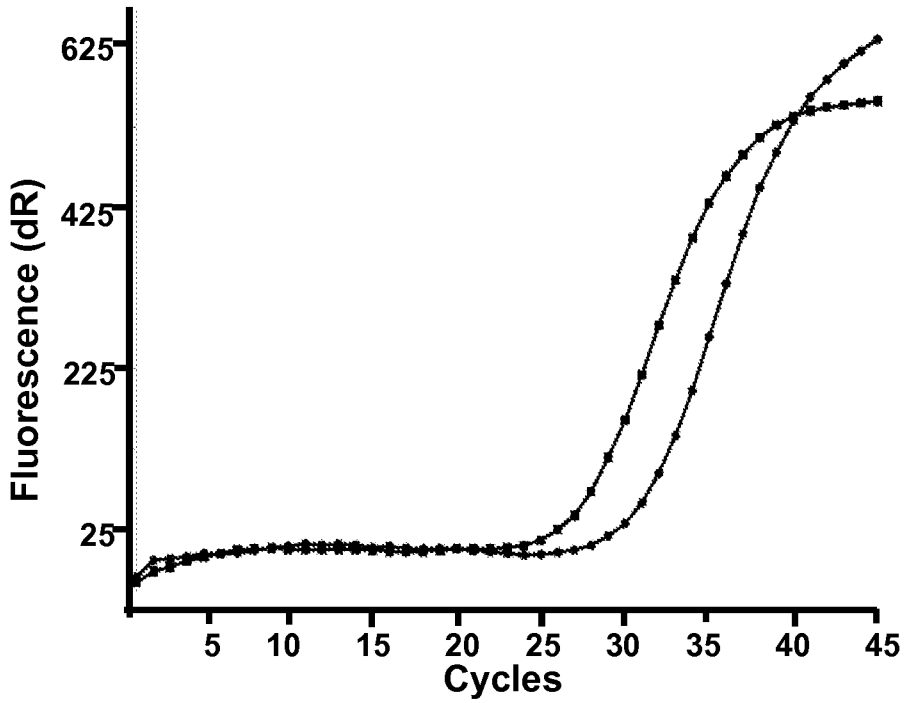

625
425
225
25
Fluorescence (dR)
5
10
15
20
25
30
35
40
45
Cycles

Fig. 15

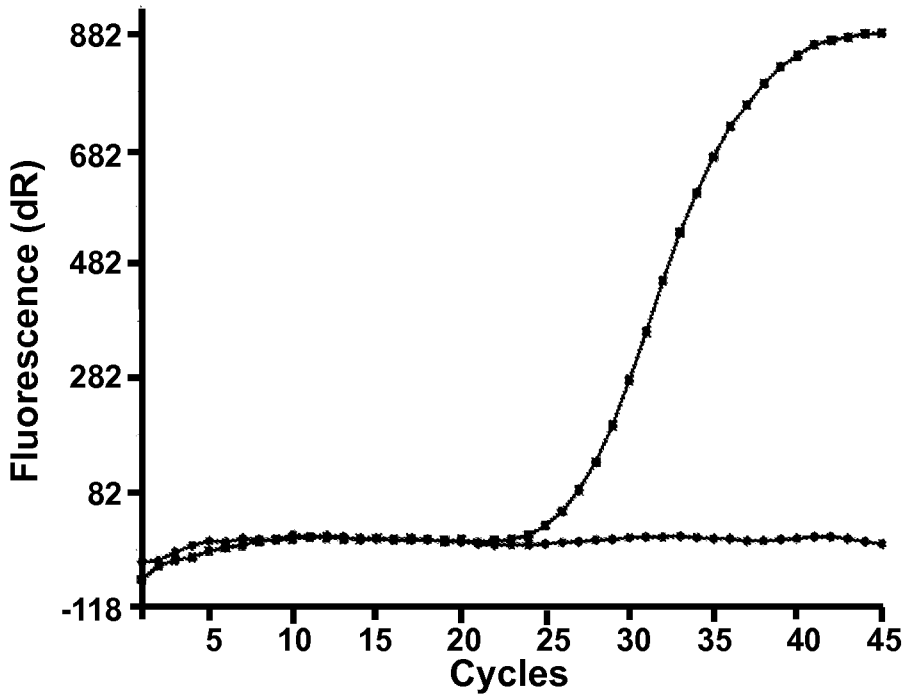


Fig. 16A

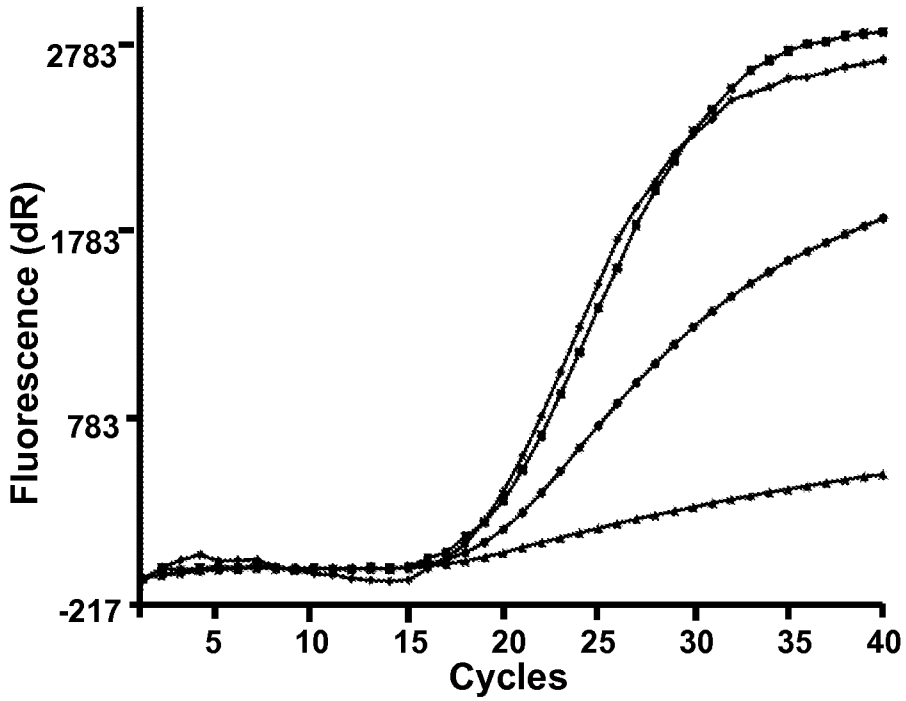


Fig. 16B

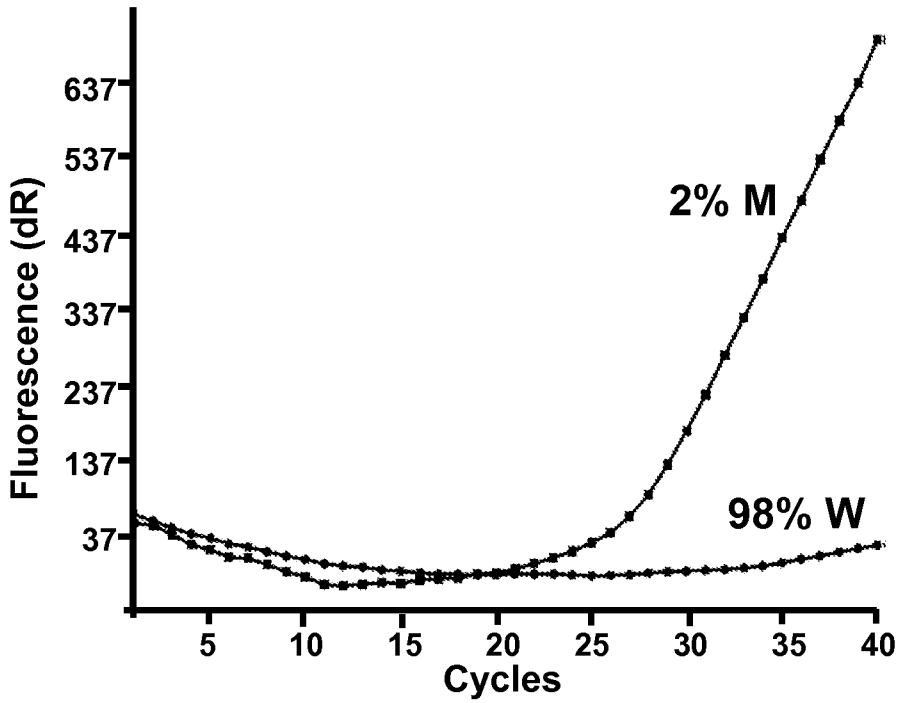

637
537
437
337
237
137
37
Fluorescence (dR)
2% M
98% W
5
10
15
20
25
30
35
40
Cycles

Fig. 17

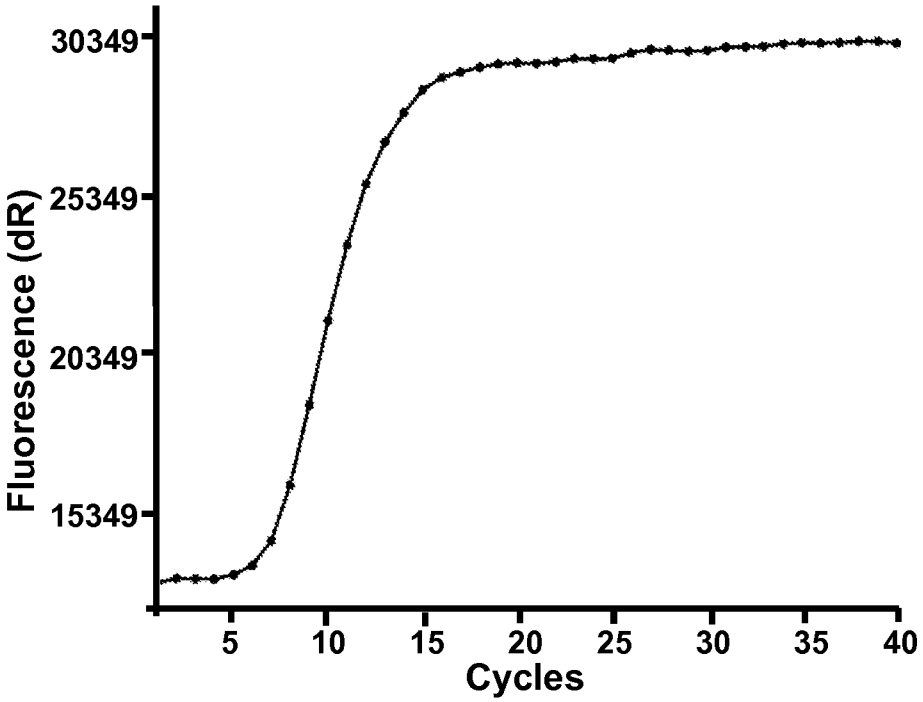

30349
25349
20349
15349
Fluorescence (dR)
5
10
15
20
25
30
35
40
Cycles

Fig. 18

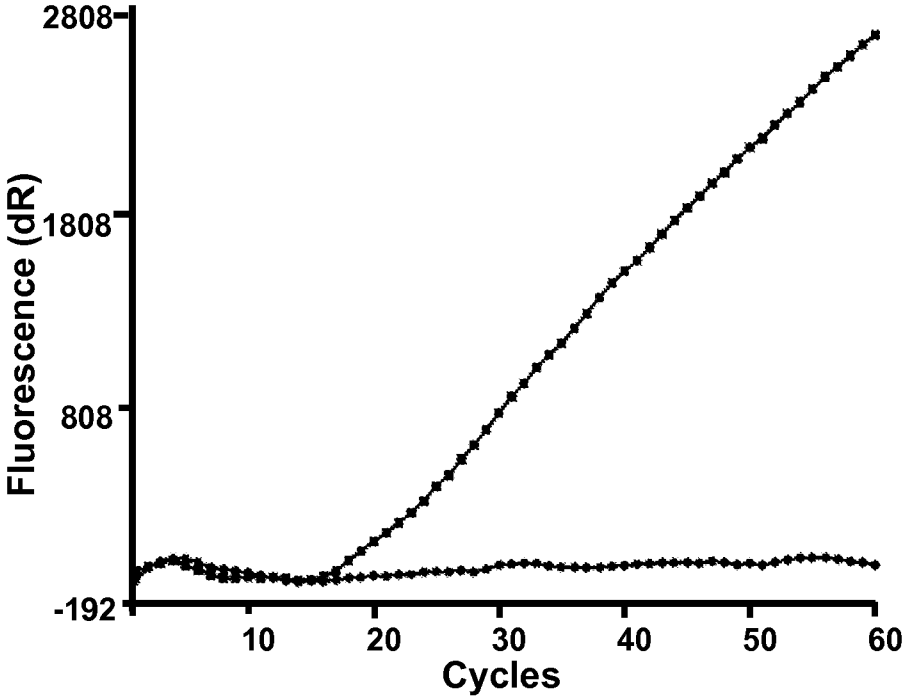

2808
1808
808
-192
Fluorescence (dR)
10
20
30
40
50
60
Cycles

Fig. 19

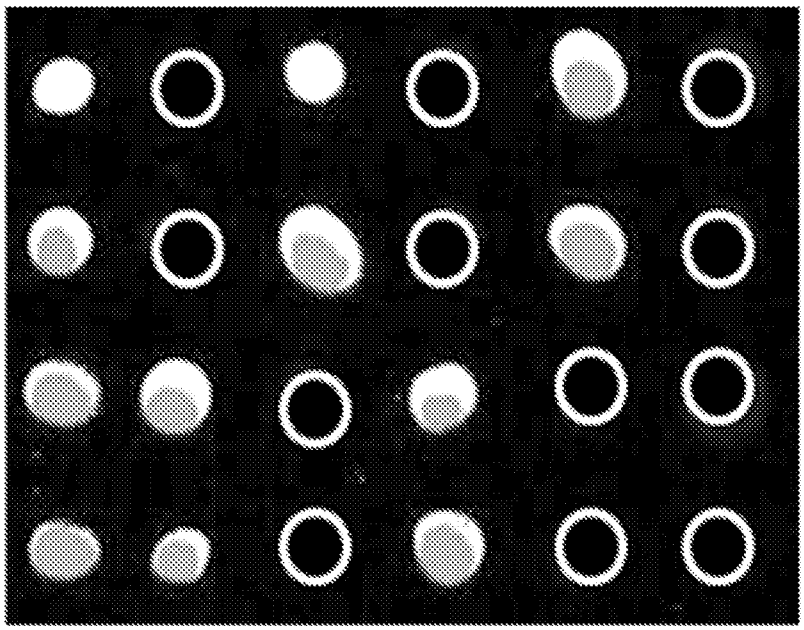


Fig. 20

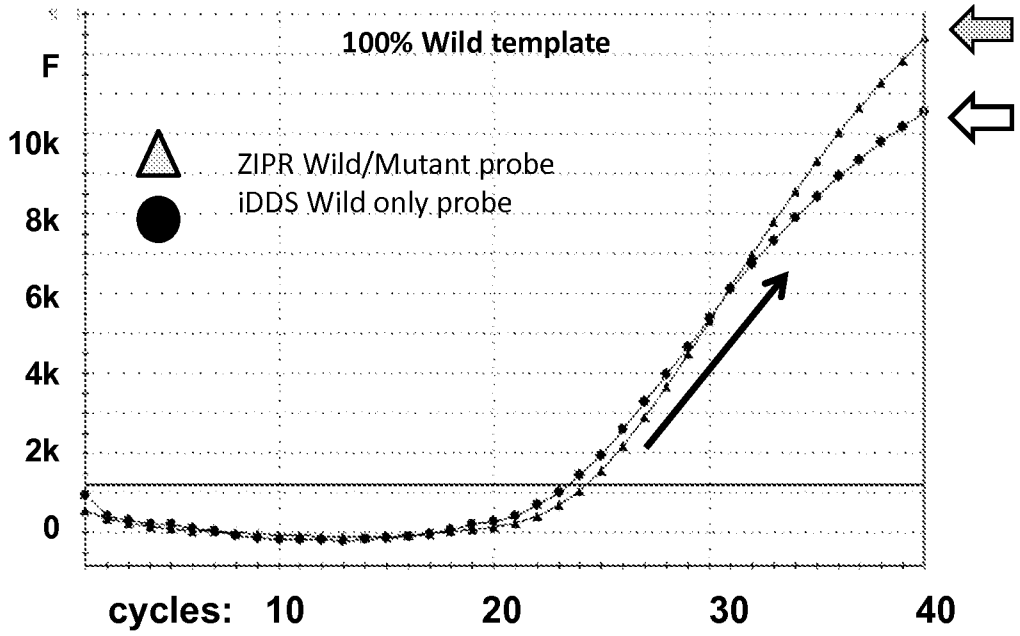

100% Wild template
F
10k
8k
6k
4k
2k
0
ZIPR Wild/Mutant probe
iDDS Wild only probe
cycles: 10 20 30 40

Fig. 21

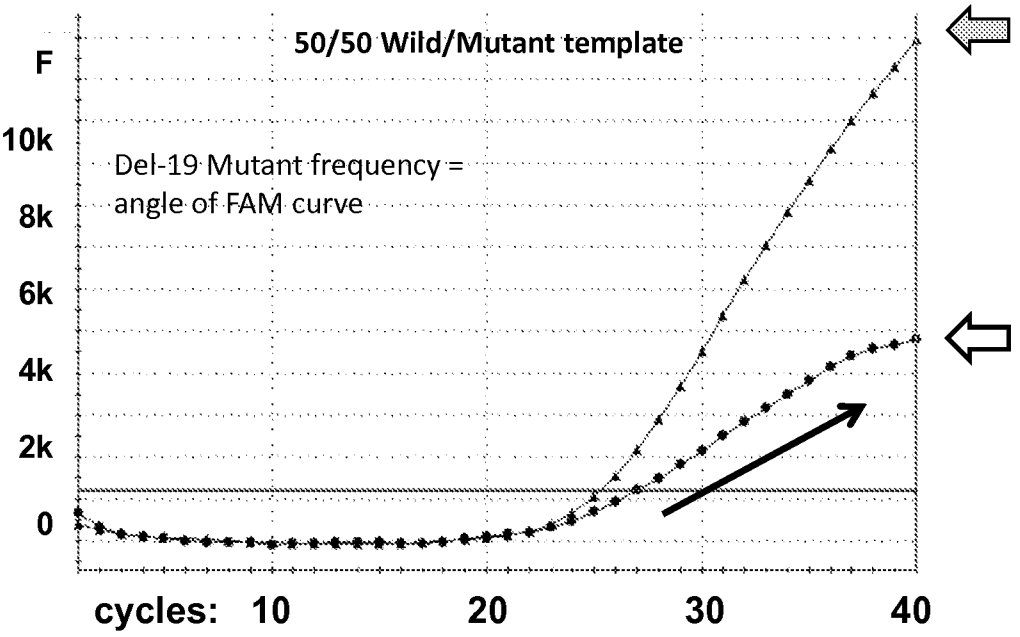

50/50 Wild/Mutant template
F
10k
8k
6k
4k
2k
0
Del-19 Mutant frequency =
angle of FAM curve
cycles: 10 20 30 40

Fig. 22

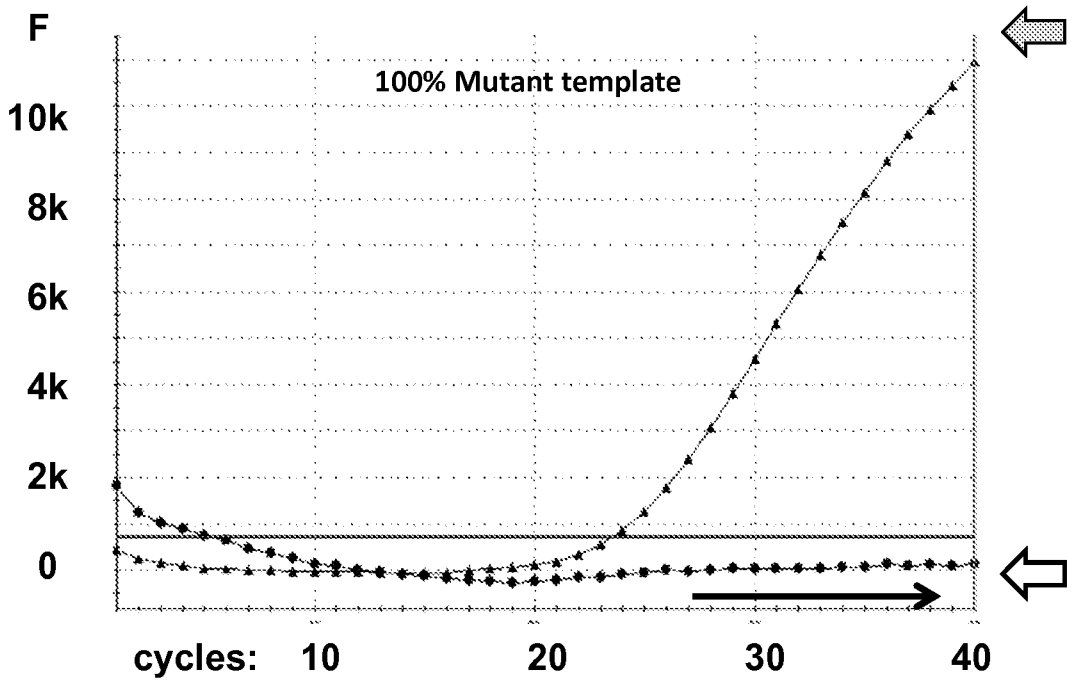

F
10k
8k
6k
4k
2k
0
100% Mutant template
cycles: 10 20 30 40

Fig. 23

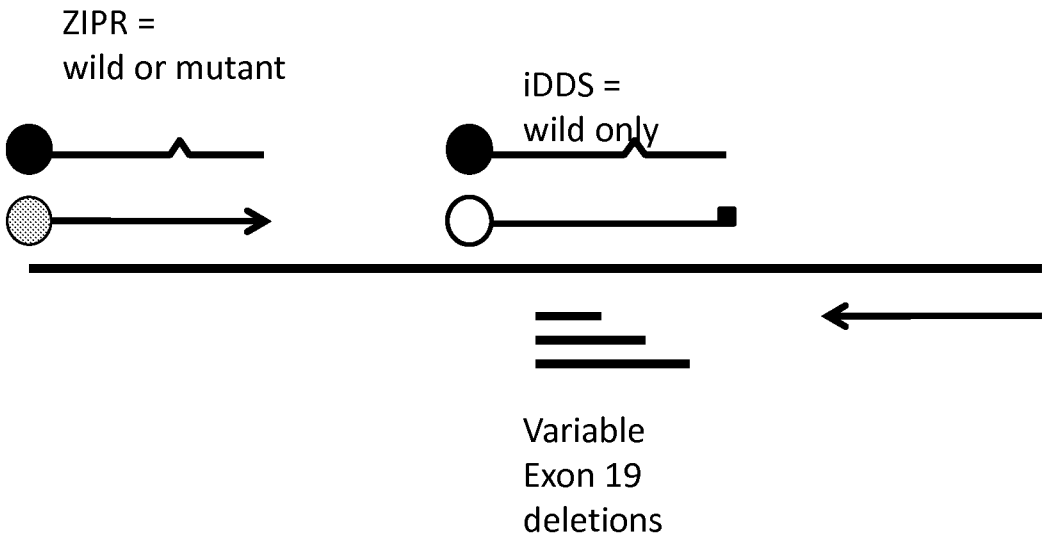
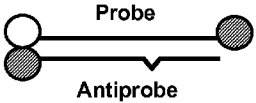
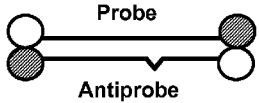
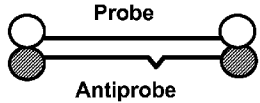

ZIPR =
wild or mutant
iDDS =
wild only
Variable
Exon 19
deletions

Fig. 24


Probe
Antiprobe


Probe
Antiprobe


Probe
Antiprobe